The effects of radiolabelled agents used in the diagnosis and treatment of cancer, on inflammatory cytokines

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Dissertation submitted in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Sciences at the North-West University

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Examination: November 2019

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My grace is sufficient for you, for my power is made

perfect in weakness

~ 2 Corinthians 12:9

PREFACE AND DECLARATION

This dissertation is written in article format, using the North-West University (NWU) guidelines and requirements, and the chosen journal guidelines were applicable.

In this project, samples from another study, that of Prof M Akerman at the University of KwaZulu-Natal in collaboration with the South African Nuclear Energy Corporation (Necsa) with Prof JR Zeevart, and the Department of Science and Technology (DST) /NWU Preclinical Drug Development Platform (PCDDP), was used. Candidate compounds are evaluated in preclinical trials, using *in vivo* models and compounds synthesised by Prof Akerman.

The tissues from these animals were used, in addition to control groups of animals, to determine the effects that radiolabelled compounds have on inflammatory cytokines, as well as oxidative stress biomarkers.

The cell culturing, animal monitoring and all post-mortem tests using the tissue and serum samples were conducted by myself, after successfully completing the appropriate courses.

Tissue samples were homogenised with the assistance of Prof R Pieters at the Unit for Environmental Sciences and Management, Faculty of Natural and Agricultural Science. Doctor Wihan Pheiffer trained and assisted me in conducting the appropriate biomarker and cytokine analysis. The cytokine analysis was done in the NWU Laboratory of Analytical Molecular Biology (LAMB), where Mr Emile Jansen van Rensburg, from the Department of Biochemistry, Faculty of Natural and Agricultural Sciences assisted me with the technical side of the software used to analyse the samples. The malondialdehyde (MDA) and protein carbonyl (PC) analysis was done with the help of Dr Ruan Gerber at the Unit for Environmental Sciences and Management, Faculty of Natural and Agricultural Sciences.

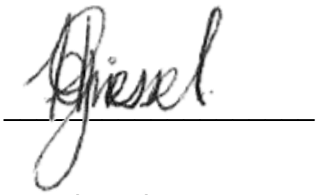
For data interpretation after analysis, the data was submitted to Prof Faans Steyn at the Statistical Consultation Services, Potchefstroom Campus, NWU, for statistical analysis.

The two chapters in this dissertation were written according to the guidelines for authors of the *Journal of Pharmacological and Toxicological Methods* and the *Journal of Immunology Research*.

Results from this study were presented at the Drug Safety Africa Conference (20 – 22 November 2018, Potchefstroom, South Africa), the Safety Pharmacology Society annual meeting (23 – 26 September 2019, Barcelona, Spain), and the Academy of Pharmaceutical Sciences South Africa (9 – 11 October 2019, Centurion, South Africa).

I, Heléne Griessel, hereby declare that this dissertation, submitted by myself, is my own work. This work has not been submitted previously to another university by me.

Signature



H. Griessel
Date: 18/10/2019

ACKNOWLEDGEMENTS

Firstly, I would like to thank my Heavenly Father for having this opportunity, the talent, capability and endurance to have fulfilled this study. I am in awe of His goodwill and grace and am abundantly blessed. God surely can turn ashes into glory.

I would then like to acknowledge the following people and institutions for each contribution.

- The DST/NWU Preclinical Drug Development Platform, in particular Prof Anne Grobler, for the privilege to complete this study and to have learnt so much and for making this possible.
- My supervisor, Dr Wihan Pheiffer, for his support, guidance, patience and expertise. I learnt so much, and are grateful for the way you made me see the positive even in bad times.
- My co-supervisor — Prof Anne Grobler was responsible for giving me this project as well as a major role in the collaboration with Necsa. She assisted in the layout of the project, overall comments and final compiling.
- My other co-supervisor — Dr Jesslee du Plessis for assisting me in the technicalities and concerns of compelling the dissertation, writing and overall language of the dissertation, your time, guidance and input in this study as well as the writing of the dissertation.
- Dr Cathryn Driver, Prof Jan-Rijn Zeevart and everyone who assisted me in this study at Necsa.
- Prof Matthew Akerman, for making this study possible by allowing me to use the treatment exposed tissues for my study and opening a new field for me.
- Mr Cor Bester, Mr Kobus Venter and the personnel of the Vivarium — thank you for your guidance and assistance in the animal handling and study.
- Prof Rialet Pieters for your assistance in the sample preparations and Dr Ruan Gerber for guiding me through the MDA and PC analysis.
- Mr Emile Jansen van Rensburg for assisting me in the technicalities and setup of the programme used for cytokine analysis.

- Mr Ndavheleseni Tshikudo and Miss Amcois Visagie from Becton Dickenson for your support, assistance and technical input with the flow cytometer and cytokine analysis.

- All my colleagues at the PCDDP, thank you for making these past two years memorable, even in the hardest times, you made it such a positive and efficient environment, and it was a great pleasure. Jaco Louw, Bisrat Bekele, and Tumelo Kgoe thank you for all the memories and motivations we were able to share.

- Bisrat Bekele, you guided me through cell culturing, animal handling and overall guidance and academic advice. I am grateful for your patience and advice, with your positive energy.

- Medicine Usage of South Africa (MUSA) for enabling me to do my academic internship.

- To the NWU and the DST/NWU PCDDP for the financial support, making this study possible.

—

- To my parents, Jacques and Wilma Griessel for making this possible, always motivating me and raising us with passion and endurance and the believe that we can conquer anything in life that we put our minds to. Thank you for always backing us in every new adventure, your never-ending support, motivation, love, and prayers. I would never be able to explain how much I appreciate it.

- To my best friend, Gerdus Bester, thank you for you never-ending support, love and motivation, for always believing in me, even when I struggled myself. I am so thankful for your positivity and for helping me to always look at the bigger and brighter side of things and teaching me how to endure in the most difficult times. Thank you for being on my side and loving me!

- To my siblings, MJ, Martin and Janaé, thank you for your love and patience, your support and to be able to dream with you about the future.

- To my second parents, Oom Jan and Henda, thank you for always believing in me, guiding me mentally as well as spiritually, for your love, support, and motivation.

- To my family and friends who have supported me through this journey, thank you for your utmost support and love. Thank you for every cup of coffee when needed, scriptures, and celebrations.

PROJECT SUMMARY

This study was conducted to determine the effects that two radiolabelled compounds have on the inflammatory cytokines and oxidative stress biomarkers in xenograft Rowett nude (RNU) rat models.

Safety and efficacy are of concern when developing new compounds. There is a paucity of information on the influence of new compounds on inflammatory cytokines in combination with oxidative stress markers. Cytokines are potential markers of drug safety, and oxidative stress markers may indicate the impact of drugs on physiological processes. There is no formal reference of normal ranges of inflammatory markers. By the establishment of a baseline for preclinical safety studies, the effects of new compounds on cytokines can be quantified. The aim of this study was to determine the changes in the cytokines and anti-oxidant enzymes in tissue and serum samples *in vivo* (xenograft rats) after treatment with radiolabelled compounds — copper (II)- and palladium (II) chelate. Tissue was collected from RNU rats from an untreated non-xenograft group and xenograft (A549 human lung adenocarcinoma) groups: a control group and two radiolabelled compound exposed groups.

Cytokines were quantified by cytometric bead array using flow cytometry. Oxidative stress biomarkers — intracellular reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and protein carbonyl (PC) — were quantified using enzymatic-based colorimetric assays.

The only significant difference in the percentage composition of cytokines was seen in the tumour tissue. Compared to the xenograft control, the copper (II) chelate treatment increased the majority of the cytokines and biomarkers in the kidneys, and decreased their levels in the liver. In contrast, the palladium (II) chelate treatment had the opposite effect in both the kidneys and the liver (i.e. decrease and increase, respectively).

It is evident from the large differences between treatment groups relative to the control that both cytokines and oxidative stress biomarkers can be used to elucidate the safety of new compounds in preclinical drug development studies.

Keywords: *Cancer, Non-small-cell lung cancer, cytokines, pro-inflammatory cytokines, anti-inflammatory cytokines, oxidative stress, cells, tumour, inflammation, immune, biomarker, reactive oxygen species, antioxidants, oxidative stress damage, lipid peroxidation,*

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ABBREVIATIONS

Units

µ	Micro
µg	Micro gram
µl	Micro litre
%	percentage
°C	Degrees Celsius
G	Gram
Kg	Kilogram
Mg	Milligram
Min	Minutes
Mm	Millimetre
mM	Micromolar
ngSOD/mg	Nanogram SOD per milligram
nM	Nanomolar
Nmol	Nanomole
pg/mL	Pictograms per mililitre
RCF	Relative centrifugal force
w/v	Weight (gram)/ volume (100 millilitre)

A

A549	Non-small-cell lung cancer cell line
AnimCareREC	Animal research ethics committee
ATP	Adenosine triphosphate

B

BD	Becton Dickenson
BMI	Body mass index

C

CO	Carbonyl
CO_2	Carbon dioxide
CAT	Catalase
Cu	Copper

D

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid

DNPH	2,4-Dinitrophenylhydrazine
DST	Department of Science and Technology
DTPA	Diethylene triamine penta-acetic acid

F

FBS	Foetal bovine serum

G

GLP	Good laboratory practice
GPx	Glutathione peroxidase
GSH	Glutathione

H

H_2DCFDA	2, 7-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HNE	4-hydroxynonenal

I

IFN-γ	Interferon-gamma
IL-1	Interleukin 1
IL-1ß	Interleukin 1 beta
IL-1ra	Interleukin 1 receptor antagonist
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-12p70	Interleukin 12p70
IL-17	Interleukin 17
IL-17A	Interleukin 17A
IL-17F	Interleukin 17F
IVCs	Individual ventilated cages

K

KCl	Potassium chloride
$KMnO_4$	Potassium permanganate

L

LAMB	Laboratory of Analytical Molecular Biology
LCC	Large cell carcinoma
LDH	Lactate dehydrogenase

M

MAP	Mitogen-activated protein
MDA	Malondialdehyde
microPET	Micro positron emission tomography
MUSA	Medicine Usage of South Africa

N

Necsa	The South African Nuclear Energy Corporation
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NSCLC	Non-small-cell lung cancer
NWU	North-West University

P

PC	Protein carbonyl
PCA	Principle component analysis
PCDDP	Preclinical Drug Development Platform
Pd	Palladium
PET	Positron emission tomography

R

RCu	Copper (II) chelate compound treatment group
RDA	Redundancy analysis
RH	Non-xenograft control group
RNU	Rowett nude
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPd	Palladium (II) chelate compound treatment group
RX	A549 xenograft control group

S

SA	South Africa
SCLC	Small-cell lung cancer
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SOD	Superoxide dismutase
SPECT	Single-photon emission computed tomography

T

Th	T helper
Tfh	T follicular helper
TCA	Trichloroacetic acid
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TNF-α	Tumour necrosis factor alpha
TNM	Tumour, Node, Metastasis
Tris	*tris*(hydroxymethyl)aminomethane

W

WHO	World Health Organization

CHAPTER 1: BACKGROUND AND SCOPE OF THE STUDY

1.1 Scope and background

Cancer is a disease that has an enormous effect on millions of people annually — a life-changing disease, which receives extensive attention as a topic of research. Cancer refers to a group of diseases where cells divide uncontrollably and potentially spread through the body. These abnormal cells may be the due to mutations that result in faulty cell growth, differentiation or division (Frederic *et al.,* 2012). Uncontrollably dividing cells can form two types of tumours — benign or malignant; however, benign tumours do not invade or infiltrate tissue nearby and consequently are not classified as cancer (Dictionary of Medical terms, 2005a; National Cancer Institute, 2005). Malignant tumours have the ability to infiltrate tissues nearby and therefore make it possible to metastasise (Dictionary of Medical Terms, 2005b; Frederic *et al.,* 2012; National Cancer Institute, 2005).

According to the Global Cancer statistics, 33 million people were living with cancer during 2012; of these 14 million were new cancer cases and eight million people died of cancer (Ferlay *et al.,* 2012). In 2018, estimations of 18 million new cancer cases and 10 million mortalities due to cancer were made (Bray *et al.*, 2018; World Health Organization (WHO), 2018), making cancer the second leading cause of death globally (Siegel *et al.*, 2019; WHO, 2018). These high, increasing numbers make the diagnosis and treatment of cancers much more urgent and therefore require new and improved diagnostic methods and effective treatments as soon as possible (Gilligan & Dwyer, 2017; Wang *et al.,* 2017). There are several treatments for cancer, usually with severe adverse effects, but new and improved treatments are necessary to decrease the number of mortality cases caused by cancer (Iqbal *et al.,* 2017). Radiolabelled compounds can be classified as drugs that contain atoms of some radioactive elements. Radiolabelled compounds deliver small amounts of ionising radiation to diseased sites in the body. They may consist of macromolecules or even small organic molecules, and is used for diagnosis or treatment of various diseases such as thrombosis, inflammation and tumours (Fichna & Janecka, 2003). What makes radiopharmaceutical compounds so desirable is the fact that these compounds can be synthesised and developed according to the pharmacokinetic requirements — for both diagnosis and targeted therapies (Blower, 2015; Fichna & Janecka, 2003; Ramdhani *et al.*, 2019). A correlation was noted between radiation therapy and changes in inflammatory markers (Bower *et al.*, 2009; Müller & Meineke, 2007). This is an indication of the importance of doing specialised tests and knowing the effects that radiolabelled compounds may have on inflammatory markers.

It is postulated that inflammation has an effect on the development of cancer, as well as the progression thereof (Brenner *et al.,* 2014; Coussens & Werb, 2002). Inflammation is defined by

Coussens and Werb (2002) as a complex network responding to tissue injury. Leukocytes – monocytes, neutrophils and eosinophils — are activated and migrate from the venous system to the damaged tissue. In this network, another essential role is played by tissue mast cells (Zhang & An, 2007).

Cytokines belong to a class of several classes of biomarkers for inflammation (Brenner *et al.,* 2014), which are soluble protein modulators secreted by immune cells in response to antigens. They are important in intracellular communication and to ensure an effective immune response. Each cytokine is unique and can affect the production and the modulation responses of other cytokines and therefore work in a complex network (Yoshimoto & Yoshimoto, 2013); each play major roles in the immune system's defence. Cytokines are produced by different cell groups, but the dominant producers of cytokines in the body are macrophages and helper T cells (Zhang & An, 2007). Cytokines can be divided into two main groups, namely anti-inflammatory cytokines and pro-inflammatory cytokines (Zhang & An, 2007). The two types of inflammatory cytokines function to control each other's responses. For instance, anti-inflammatory cytokines have to control the response of pro-inflammatory markers and *vice versa* (Zhang & An, 2007). An imbalance between these cytokines is responsible for inflammation (Coussens & Werb, 2002), which can enhance cell growth, differentiation of cells, as well as the survival and apoptosis of cells that can cause tumour cells and result in cancer (Lu *et al.*, 2006).

Different ranges of what are indicated as normal ranges of pro- or anti-inflammatory cytokines have been published (Chapman *et al.*, 2010; Keeley *et al*., 2014; Marques-Vidal *et al.*, 2011; Soto-Méndez *et al.*, 2015; Wyczalkowska-Tomasik *et al.,* 2016). This is because inflammatory markers are influenced by population, environment, gender, body mass index (BMI) and age (Marques-Vidal *et al.*, 2011). Each study taking place in a different setting or with subjects that differ will have its own results in terms of the ranges of inflammatory markers (Chapman *et al*., 2010; Keeley *et al*., 2014; Marques-Vidal *et al.*, 2011; Soto-Méndez *et al.*, 2015; Wyczalkowska-Tomasik *et al*., 2016). For this reason, it is imperative to close the gap on the lack of knowledge of 'normal' ranges of cytokines and to set baselines for different conditions. In South Africa (SA), there are no data available on the anti-inflammatory or pro-inflammatory cytokine values in healthy people or what the reference of normal ranges of inflammatory markers may be.

Another important marker in cancer is the secondary messenger — oxidative stress, which regulates physiological processes (Filaire *et al.*, 2013). Oxidative stress is an important factor in both acute and chronic diseases (Ceconi *et al.*, 2003; Dalle-Donne *et al.*, 2006; Sies, 1997). Cancer is one disease that may be caused by oxidative stress, as *in vitro* studies in cell cultures done by Dalle-Donne *et al.* (2006), indicated.

According to Sies (1997), oxidative stress can be defined as the imbalance between antioxidants and oxidants in favour of oxidant levels (Ďuračková, 2010; Sies, 1997). If antioxidant defences do not result in the inactivation of reactive oxygen species (ROS), oxidative stress will increase (He *et al.*, 2017) and cause oxidative stress damage, leading to cell, tissue and DNA damage (Kousteni, 2011; Okada *et al.*, 1999).

The problem with an increase in oxidative stress is the damage it causes to proteins, lipids and nucleic acid bases, which, in turn, can compromise cell health. In theory, any oxidative damage can then contribute to the development of a disease (Dalle-Donne *et al.*, 2006). The biomarkers of oxidative stress are indicators of changes in the physiological state in a disease at that point in time (Dalle-Donne *et al.*, 2005).

Both the levels of selected cytokines and oxidative stress contribute to cancer, but a definite correlation with cancer has not been made yet (Rahman & MacNee, 2000; Wu *et al.*, 2013). Oxidative stress and inflammatory markers seem to have an effect on each other (Biswas, 2016), and possible pathways were suggested (Anderson *et al.*, 1994; Flohé *et al.*, 1997), but more studies in this field are needed to confirm these suggestions.

As humans do not present themselves prior to development of a disease for clinical investigations, it is not possible to do certain studies involving the development and changes associated with diseases in humans. A strong justification will furthermore be needed to categorise blood collection at several time points from healthy humans or to put them under certain stressors to evaluate and analyse behaviours, molecular changes and physiology.

Animal models have similarities to humans' histological and clinical features (Lu *et al.*, 2014). To be able to test hypotheses at a living organism level and also to validate human data, it is necessary to use animal models (Barré-Sinoussi & Montagutelli, 2015). These models also have similar inflammatory profiles than those of humans; they are reproducible and inexpensive, and they respond in a very similar manner to therapeutic drugs than humans do (Lu *et al.*, 2014). Therefore, it is appropriate to make use of animal models in studies involving cancer and the analysis of potential therapies.

1.2 Research problem

The increasing number of cancer cases reported annually underlies the urgency of developing new and improved drugs for the treatment, but also for the diagnosis of cancer. In the development of new compounds, the focus of preclinical studies is usually the bio-distribution, toxic effects, pharmacokinetics and pharmacodynamics of the candidate drugs. According to the literature, radiation therapy is associated or correlates with changes in inflammatory markers (Bower *et al.*, 2009; Dovšak *et al*., 2018), and treatments have been shown to influence oxidative

stress in cells. A correlation between oxidative stress and inflammatory markers has been found (Biswas, 2016; Grivennikov *et al.*, 2009); therefore, it is essential to investigate the effect of these radiolabelled compounds on inflammatory markers and oxidative stress biomarkers, in the most prevalent cancers such as lung cancer. It is important to do preclinical analyses on the effects that these new compounds may potentially have on cancer patients, as further compromise of the immune system must be avoided, and inflammatory cytokines and oxidative stress biomarkers are indicators thereof.

1.3 Aim and objectives

1.3.1 Research aim

The research aim for this study was to establish the effects that two radiolabelled compounds – a copper (II) chelate and a palladium (II) chelate – may have had on cancer-relevant inflammatory markers and oxidative stress biomarkers.

1.3.2 Research objectives

In order to meet the research aim, the following study objectives were set:

- To establish a baseline for cytokines in immuno-deficient Rowett nude (RNU) rats that had undergone no stressors (absolute control).
- To determine the cytokine levels relevant to non-small-cell lung cancer (NSCLC) (represented by the A549 cell line) in an established xenograft RNU rat model.
- To determine the cytokine levels after radiolabelled (i) copper (II) chelates and (ii) palladium (II) chelates exposure in A549 xenografted RNU rat tissue and serum.
- To determine the effects of (i) copper (II) chelate compounds and of (ii) palladium (II) chelate compounds on oxidative biomarkers in A549 xenografted RNU rat tissue and serum.

1.4 Study design

The animal study commenced, after ethics approval from the NWU Animal Research Committee (AnimCareREC) [NWU-00178-18-A5] (Appendix A). Samples collected during the animal study were analysed for selected cytokine and oxidative stress marker levels, to indicate the effects of radiolabelled compounds. Four RNU rat groups were used, a non-xenograft rat group (RH) serving as a control; a xenograft group (RX) serving as a cancer control; a copper (II) chelate compound treatment group (RCu) and palladium (II) chelate compound treatment group (RPd), both serving as the experimental groups. The effect on both pro- and anti-inflammatory markers were analysed. The oxidative stress markers measured were reactive oxygen species (ROS), catalase (CAT), superoxide dismutase (SOD), protein carbonyl (PC) and malondialdehyde (MDA). Statistical analysis was done on the results, after which the data were interpreted and conclusions made. For additional information on the group allocation and animal study please refer to Appendix B.

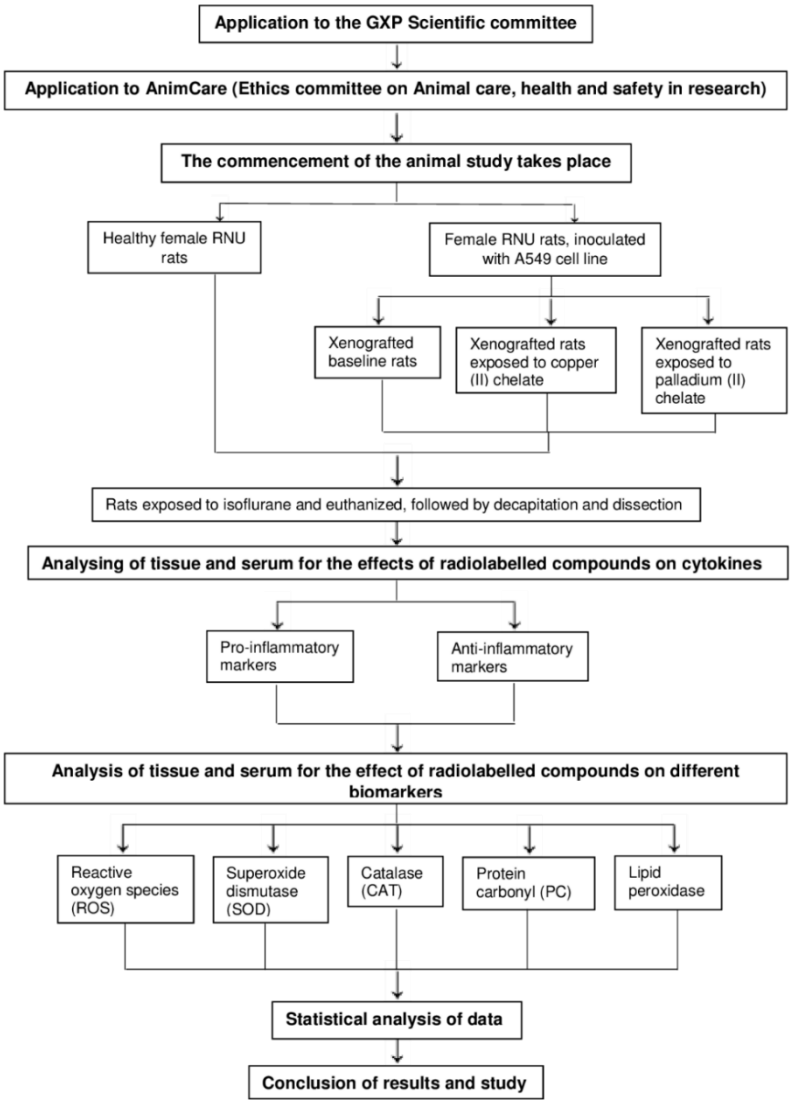

Figure 1: A layout of the study design indicating the course of the study — animal study, analysis of cytokines and oxidative stress biomarkers, statistical analysis and conclusion of the study

1.5 Structure of dissertation

Compilation of chapters

This chapter **(Chapter 1)** provides a brief overview of the background, justification and scope of the study. This chapter also includes the formulated research problem, aims and objectives, study design and outline of the chapters.

Chapter 2, is a literature study written in a review article format. This chapter details the cancer of interest in this study, relevant inflammatory markers, biomarkers of oxidative stress. Finally, the relationship between oxidative stress markers and inflammatory cytokines will be tested. This chapter is written in accordance with the author guidelines of the *Journal of Immunology Research*.

Chapter 3 is presented as a research manuscript, briefly discussing the literature regarding inflammatory markers, oxidative stress biomarkers, and radiolabelled compounds relevant to cancer. The effects of these compounds on inflammatory markers and oxidative stress biomarkers were determined and are presented. This chapter is written in accordance with the guidelines for authors of the *Journal of Pharmacological and Toxicological Methods*. This manuscript will be considered for publication.

Chapter 4 is the concluding chapter of the study. The project results are summarised and the final discussion and conclusions are made. Recommendations and future prospects for further research are also included.

References are provided at the end of each chapter. Article chapters will follow journal referencing styles, otherwise the NWU Harvard style was followed according the NWU Referencing Guide.

Appendices are included at the end of the dissertation. This section provides additional information on the project not included in the main body of the dissertation.

1.6 Study dependency

This study was dependent on another study (NWU-00251-17-A5), which focused in more detail on the radiolabelled compounds. In the current study (NWU-00178-18-A5), a baseline for healthy animals, as well as xenograft animals (A549) was used. As part of the effort to reduce the number of animals used, tissue and serum samples from study NWU-00251-17-A5 were used as experimental groups.

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CHAPTER 2: LITERATURE STUDY IN THE FORMAT OF A REVIEW ARTICLE

Chapter 2 is written in the format of a review article according to the guidelines for authors of the *Journal of Immunology Research.* The Author Guidelines (https://www.hindawi.com/journals/jir/guidelines/) are given in Appendix C. However, for ease of reading, the tables and figures are inserted at their appropriate positions in the manuscript. This manuscript has not been submitted for publication.

THE IMMUNE RESPONSE IN CANCER: EFFECTS OF CYTOKINES AND OXIDATIVE STRESS

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Abstract

Cancer is the result of defective processes — pathophysiological and physiological — where normal cells develop into a malignant tumour. Inflammation consists of a complex network of pro- and anti-inflammatory markers that have an influence on each other. This complex network is not yet fully understood. Chronic inflammation can be causative of cancer, since there is a dysregulation of the immune system. Because cytokines can either promote or reduce tumour growth — depending on inflammatory class — they are essential to monitor in the tumour environment. Oxidative stress also plays a crucial role in physiological processes, and an imbalance between reactive oxygen species (ROS) and antioxidants results in elevated oxidative stress levels, which will cause oxidative stress damage in cells. There is a fine line in the balance between ROS and antioxidants for normal cell function and dysregulation.

Oxidative stress and cytokines each correlate with different factors in different diseases, but a definite correlation between these two factors in cancer is unclear. Nuclear factor kappa light chain enhancer of activated B cells (NF-κB) is an important player in this relationship, where it is involved in gene transcription of genes that encode both growth factors and inflammatory markers. These findings should be further investigated, as definite correlations between cytokines, oxidative stress biomarkers, and other cell mediators and factors are much more complex.

Keywords

Antioxidant, Biomarker, Cancer, Cytokines, Oxidative stress,

Abbreviations

CAT – catalase, Cu – copper, IL – interleukin, IFN – interferon, TNF– tumour necrosis factor, MDA – malondialdehyde, NFκB – nuclear factor kappa light chain enhancer of activated B cells, NSCLC – non-small-cell lung cancer, PC – protein carbonyl, Pd – palladium, , ROS – reactive oxygen species, SOD — superoxide dismutase, WHO – World Health Organization.

Introduction

Annually, millions of people are affected by cancer, whether the affect is on themselves, or those around them [1]. According to published research, cancer is a more complex disease than expected, wherein normal cells are changed into a malignant tumour in a multistage process [1, 2]. Cancer is the result of faulty physiological and pathophysiological processes in a specific area of the body [3]. These processes can become defective due to external factors such as exposure to carcinogens [1].

The immune response is the body's defence system to protect itself from stimuli that may cause it harm. It protects against changes in molecular structures and prevents changes in both the physiological and pathophysiological systems [4]. Changes in the immune system will influence the tumour microenvironment [5].

A pathological environment can arise from chronic inflammation, where cancerous tissue and eventually tumours can develop [5, 6], and which results in a necessary immune response. Both pro- and anti-inflammatory cytokines play a vital role in the complex immune response network to maintain a balance, as well as to eliminate external stimuli that might irritate or damage the body and its physiological processes [7]. Each cytokine or inflammatory marker is unique, and has its own role to play in the complex network — immune defence [7] — either by inhibiting the inflammation of the inducing reaction or by its effect on the functioning of other cytokines [8].

As a secondary messenger, oxidative stress is important for physiological processes' regulation [9, 10]. Oxidative stress is important for proliferative signalling pathways, survival and apoptosis of cells [9]. The result of an imbalance of reactive oxygen species (ROS) and its regulatory antioxidants (to the benefit of ROS) is oxidative stress, which may lead to oxidative stress damage, such as lipid, protein damage or DNA damage [11–13].

Free radicals and ROS are produced as by-products of cellular metabolism and external stimuli [14, 15]. These reactive compounds alter different pathways and cellular functions, such as redox modification and redox signalling. ROS activates inflammatory responses through the activation of cell signalling, and influences the secretion of cytokines [16, 17].

As defence system, the body produces endogenous antioxidants, which counteract the damage of ROS. These antioxidants include enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) [18, 19]. A harmony between ROS and the antioxidants is of the utmost importance, because an imbalance can result in several diseases and malfunctioning of cells

[20]. The increase in ROS levels may lead to oxidative stress damage in the form of lipid peroxidation, protein or DNA damage [21], among others.

According to the literature, the properties of inflammatory cytokines differ in the presence of cancer compared to controls [22, 23]. It has also been reported that, in cancer, the balance between ROS and antioxidants shifts to the benefit of ROS [24, 25].

The changes in these biological markers in the presence of cancer may have indicative value as to finding a correlation between the pathophysiology of a disease, with a focus on cancer. However, little to no information about such a correlation between cytokines and oxidative stress markers is available. This literature review aims to assemble the existing knowledge to bridge this gap.

Cancer

As early as 1863, Rudolf Virchow suggested that there is a connection between chronic inflammation and cancer, as cancer occurred in sites of chronic inflammation [8]. When a pre-cancerous lesion progresses into a malignant tumour, the changes in cells can be the result of genetic factors interacting with external factors, such as carcinogens. The World Health Organization (WHO) names three groups of carcinogens that can play a role in this interaction, namely biological (infections from bacteria, parasites and viruses), chemical (aflatoxin, arsenic, asbestos and tobacco smoke components), and physical (ionising radiation and ultraviolet) [1].

The stages of development in cancer can be determined by using the TNM (tumour, node, metastasis) system as provided in the International System for Staging Lung Cancer [26]. Cancers can be staged accordingly from stage 0 to stage IV [26, 27], which are dependent on the size, location, the presence and location of the lymph nodes involved, the extent of the primary tumour, and the absence or presence of distant metastatic disease [26, 27]. In lung cancer, if the tumour is present in one lung, and did not spread to distant organs or lymph nodes, it is classified as stage I. Stage II is where cancer has spread to the lung's lymph nodes, and stage 3 is where cancer did not spread to distant organs, but has spread to central chest lymph nodes. Stage 4 is present when the cancer has spread throughout the entire body [28].

The tumour environment is affected by inflammatory cells, which can initiate differentiation, growth and migration of the tumour cells, and also contributes to angiogenesis and DNA damage [5, 6]. Chronic ulcerative colitis is a chronic inflammatory bowel disease that arises from colon carcinogens, and is evidence to back the fact that chronic inflammation has a strong association with malignant diseases [5].

Over several decades, lung cancer has been the most common cancer diagnosed [29] and, according to the World Health Organisation (WHO), in 2018, lung cancer was responsible for 11.6% of new cases of cancer diagnosed and 18.4% of all cancer-related mortalities [1], making lung cancer the most prevalent cancer type [30]. There are two classes of lung cancer — non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC) [9, 31].

Non-small-cell lung cancer

Non-small-cell lung cancer (NSCLC) accounts for the majority of lung cancers, accounting for 85 to 90% of all lung cancers diagnosed [32–34].

This cancer can be caused by the effects of smoking, secondary smoke, genetics and asbestosis [33]. The problem with lung cancer is that it does not present symptoms at the early stages, and once symptoms do occur, late stage cancer is probably already present [35], and this is partly because medical organisations do not recommend routine screening for lung cancer [36].

During a study done at the Tygerberg Academic Hospital in South Africa (SA), most patients who were newly diagnosed with NSCLC already presented with signs of the metastatic disease (stage IV) (68.7%), and 24.7% had locally advanced stage III [37].

Non-small-cell lung cancer can be divided into several subtypes, such as squamous cell carcinoma, adenocarcinoma, large cell carcinoma, adenosquamous carcinoma and sarcomatoid carcinoma [34, 38, 39]. Further classification of NSCLC into subtypes has contributed to better therapy and treatment options, as the classification enables the eligibility of certain types of therapeutic strategies [39–41]. The different NSCLC groups are all grouped together because of the similarities between prognosis and treatments [38]. The classification of these different types of NSCLC and the improvement in different treatment options contribute to therapies appropriate for specific histology types [38–40].

Immune responses in cancer

An immune response is necessary for protection against immune cell infiltrations, tumours, autoimmune diseases and infectious agents, and cellular and molecular complexes are needed for the development of this response [4, 21]. A dysregulation of inflammatory markers may cause cancer [3]. Since cancer may not only be due to genetic makeup, but also due to chronic infection or a continuous exposure to irritants, an extended process of cell damage and healing may result in chronic inflammation [21, 42].

Inflammation facilitates several types of cancer [6] through etiologic pathways, and is a key feature in the development, promotion and progression of tumours [6, 42–44]. Inflammation is complex,

as some molecules involved in cancer can promote, as well as supress cancer. These molecules include reactive nitrogen species (RNS) and reactive oxygen species (ROS), chemokines, cytokines and prostaglandin [45]. The microenvironment of tumours is rich in cytokines and inflammatory enzymes, but differs between different tumours [42, 43, 46].

Several biomarkers can be inflammation related, such as mediators (growth- and transcription factors), acute phase proteins, cytokines or chemokines, RNS and oxygen species, cyclooxygenase-related factors and prostaglandins, and effectors that are immune related [6]. To be reckoned as a novel biomarker, it is important for biomarkers to be specific and sensitive [47].

Biomarkers such as inflammatory markers and oxidative stress-related molecules can be indicative of abnormal process, or of a condition or disease — such as chronic inflammation and cancer [22, 48]. Tumour cell growth may also be promoted by cytokines that, in turn, are partly being produced in the tumours [48]. In a study done by Keeley *et al.* [22], the majority of the biomarkers tested were much higher in patients with lung cancer than in those of the control groups. Therefore, the assumption is that inflammation markers will be higher in patients with cancer than those without.

Cytokines

Cytokines are soluble protein modulators, secreted by immune cells in response to antigens, and comprise several modulators, namely lymphokine, chemokine, interleukins and monokines [49]. They are important in intracellular communication as well as to ensure effective immune responses. Each cytokine is unique and can affect the production and the modulation responses of other cytokines and therefore work in a complex network [7] that can affect the differentiation, proliferation, survival, growth, movement and mutation of the stromal cells, as well as tumour cells [8].

Cytokines are produced by different cell groups, but the dominant producers of cytokines in the body are macrophages and helper T cells [6, 49]. T helper (Th) cells are mainly divided into two groups — Th1 and Th2. Cell-mediated immunity is promoted by Th1 and assists in clearing intracellular pathogens. Humoral immunity is promoted by Th2, which is responsible for protection against extracellular invaders [50]. In addition, Th17 and T follicular helper (Tfh) cells are important in cell immunity [50].

Furthermore, cytokines can be defined as anti-inflammatory cytokines and pro-inflammatory cytokines [49], based on their mechanism of action. The two types of inflammatory cytokines have a feedback control for each other's responses, and therefore they are self-limiting. For instance, anti-inflammatory cytokines have to control the response of pro-inflammatory cytokines and *vice versa* [49]. Cancer development and progression involve mainly the pro-inflammatory cytokines,

and anti-inflammatory cytokines can possibly prevent the cancer to reoccur [51]. Cytokines can contribute to the development of tumours in different ways — promoting cell growth stimulation and differentiation, as well as inhibiting the apoptosis of abnormal or altered cells [52], as well as activating mitogenic signalling pathways [21].

The accessibility and the ability to quantify cytokines make them good candidates for use as biomarkers for different diseases, including cancer [47].

Interleukin-1ß (IL-1ß)

Interleukin 1 beta (IL-1ß) is a member of the interleukin-1 (IL-1) family [53]. It is a prototypic pro-inflammatory cytokine (Table 1) that can have an effect on several cell types. The producers of this cytokine are B lymphocytes, macrophages, monocytes, natural killer cells and dendritic cells [53, 54]. After stimulation of this cytokine, its release follows typically over the following 20 to 40 hours. IL-1 promotes inflammation, regulates immune responses and promotes angiogenesis and tissue remodelling [55, 56].

There is a possibility that IL-1 may work together with the tumour necrosis factor (TNF). There is an absence of IL-1ß in healthy subjects' blood circulation [53]. In a study by Barrera *et al.* [57], higher levels of IL-1ß were found to be associated with lower haemoglobin levels and with non-smokers. Patients who smoke tend to have higher levels of IL-1ß [22]. In cancer models, IL-1 promotes angiogenesis, the growth, and metastasis of tumours [58].

Interleukin 2 (IL-2)

Interleukin 2 (IL-2) is a pro-inflammatory cytokine (Table 1), produced by Th1 cells [23]. This cytokine is required for the production of TNF-α and interferon gamma (IFN-γ) through the activation of T cells [4].

IL-2 stimulates the lymphokine-activated killer cells that boost the natural killer compartment, which, in turn, inhibits tumour growth [57, 59]. It can therefore act as a negative regulator [60] in the immune response and inhibit Th17 differentiation [61].

Treatment with low doses of IL-2 enhanced anti-tumour immunity [62], but treatment with higher doses of IL-2 was not sufficient to inhibit tumour growth [62], and did not result in better survival rates in malignant cancer, but rather induces severe adverse events in patients [63, 64]. Low dosages of IL-2 therapy did not have severe adverse events, such as pulmonary oedema, compared to high dosages (23 times higher) [65]. The suppression of this cytokine results in decreased survival rates in patients with NSCLC [66].

Interleukin 6 (IL-6)

Interleukin 6 (IL-6) is a multifunctional, immunoregulatory cytokine [67, 68]. IL-6 may be an anti-inflammatory and pro-inflammatory cytokine (Table 1), depending on the microenvironment [49, 67]. Interleukin 6 is associated with higher neutrophil counts, a higher platelet/lymphocyte ratio, low haemoglobin levels, hypoxia and hypoalbuminemia, and it plays a role in the regulation of IFN-γ [57]. IL-6's prolonged action has been shown to have the same anti-inflammatory effects as those of IL-10 [69].

The conclusions drawn from several studies on IL-6 are contradictory — Enewold *et al.* [70] stated that high levels of IL-6 were associated with lung cancer prognosis and better survival; however, Silva *et al.* [71] showed that overall survival in patients with high IL-6 plasma levels was worse than those with low levels of IL-6. [72], indicating that diagnosed lung cancer patients had high levels of IL-6, which was confirmed by Chang *et al.* [73], who reported that IL-6 in high levels correlated with poor survival and response to treatment. Elevated levels of IL-6 in patients without diagnosed cancer were found in patients subsequently diagnosed with lung cancer [74], and those with diagnosed lung cancer had high levels of this cytokine, and tumour progression was enhanced by IL-6 [72].

Interleukin 8 (IL-8)

Interleukin 8 (IL-8) is a pro-inflammatory cytokine (Table 1) that also induces angiogenesis [75]. IL-8 is known as an autocrine growth factor for cancer cells [76] and is induced by IL-1 [58]. IL-8 is associated with lower haemoglobin levels, higher counts of leucocytes and neutrophils, and also fatigue [57]. According to Seike *et al.* [23], significantly higher levels of IL-8 were present in tumour tissue compared to the levels found in noncancerous tissue, and that IL-8 has an essential role in the growth of tumours [58; 75]. This was confirmed by a human study that showed an increase in IL-8 levels in patients with lung cancer, compared to those of the controls [74]. Patients whose tumours expressed IL-8 levels similar to the median or greater showed worse survival than those whose levels were under the median [23].

Interleukin 10 (IL-10)

Interleukin 10 (IL-10) is an immunosuppressive, anti-inflammatory cytokine (Table 1) [77, 78]. Initially, IL-10 was thought to be produced by Th2 cells [79] and to inhibit the function of Th1 cells, but it is now recognised that IL-10 is produced by a majority of immune cells — T cells, B cells, macrophages and dendritic cells [77].

Interleukin 10 can only be produced if the immune cells are stimulated [77]; as a result, it regulates the humoral immunity [23]. This cytokine results in the direct suppression of immune cells and in the inhibition of the activation and effector functions of T helper cells secondarily. This can lead to a decreased immune response. It can impair the immunostimulatory secretion of cytokines and act as immunoregulatory molecule [66]. Therefore, the regulation of IL-10 is essential as there must be sufficient IL-10 to inhibit an excessive immune response, but it should not be present at such a high level that it damages the protective immune response [77], because the absence of IL-10 has shown that aggravated chronic inflammation occurs [78].

It is stated that IL-10 can suppress Th1 cells that produce IFN-γ [79] and, according to Huber *et al*. [80], Th17 cell activity can be directly inhibited by IL-10 acting on the T cells. Interleukin-10 also has potent anti-inflammatory effects and can repress the expression of IL-1, IL-6 and TNF-α by activated macrophages [49]. A decrease in survival was correlated with increased levels of IL-10 in patients with NSCLC, and it suppressed IL-2 levels in a dose-dependent manner with up to 85%, and inhibited IFN-γ by 90% [66].

Interleukin 12 (IL-12)

Interleukin 12 (IL-12) is an essential immunoregulatory cytokine that results in a pro-inflammatory response (Table 1) [81]. It is primarily produced by antigen-presenting cells or by stimulated macrophages, which have a short half-life *in vivo* [82]. During infections, phagocytes and dendritic cells are responsible for producing IL-12 in response to pathogens [81]. Innate responses are regulated during infection by the expression of IL-12, which determines the adaptive immune response. IFN-γ can also be induced by IL-12 [81; 82] through its action on T cells. Patients who had elevated levels of IL-12p70 were later diagnosed with lung cancer [22].

Interleukin 17A (IL-17A)

Interleukin 17A (IL-17A) is a pro-inflammatory cytokine (Table 1) that is part of the interleukin 17 family and produced by the most recent described T helper cell — Th17 [83; 84]. In the IL-17 family, IL-17A and interleukin 17F (IL-17F) showed the most similarities, as these two cytokines bind to the same receptors, namely IL-17 receptor A and IL-17 receptor C. After pro-inflammatory stimulation, IL-17A and IL-17F can both be rapidly produced. These two cytokines induce the production of other pro-inflammatory markers [84]. Both IL-1 and IL-6 are necessary to initiate Th17 expression, after which Th17 regulates the expression of IL-17A [85].

Studies about the role of Th17 and especially IL-17A show contradictory results. IL-17A was correlated with the prognosis of advanced stages of lung cancer [71], but, in contrast, Martin-Orozco *et al*. [50] showed that tumour development is prevented by Th17, where the CD8+ T cells are activated. The latter confirms that a protective role against tumours is played by Th17 and IL-17A [50].

These contrasting statements were clarified when it was discovered that if lymphocytes are present in the tumour environment, tumour rejection is promoted by IL-17, but when lymphocytes are absent, IL-17 promotes angiogenesis and tumour growth [50; 86]. Therefore, it could be concluded that IL-17A has both pro- and anti-tumour activities, depending on the type of cancer, as well as the stage of the diagnosed cancer [84].

Tumour necrosis factor alpha (TNF-α)

Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine (Table 1) and is the cytokine that is released the most rapidly of all cytokines [44; 87]. TNF-α has lowering effects on IL-1ß and IL-6; if TNF-α is reduced, IL-1ß and IL-6 levels are also reduced. TNF-α, in particular, is responsible for this reducing activity [87], which is specifically produced by Th1 [23].

This inflammatory marker is the key molecule in mediating inflammatory processes that contributes to tumour progression. TNF-α can be produced by astrocytes, fibroblasts, smooth muscle cells, Kupffer cells, tumour cells, keratinocytes and macrophages [88].

If TNF-α is chronically produced, it may act as a tumour promoter (endogenously), which assists in the remodelling of the tumour, as well as the development of stromal cells, which are needed for the tumour to grow and metastasise [8].

Interferon-gamma (IFN-γ)

Interferon-gamma (IFN-γ) is produced by Th1 cells and natural killer cells. According to a study performed by Mühl and Pfeilschifter [89], this pro-inflammatory cytokine also has anti-inflammatory properties (Table 1). Whether interferon is pro- or anti-tumorigenic will depend on the cancer type, stage and microenvironment of the tumour [90]. This cytokine has the potential to inhibit IL-1 and IL-8 and therefore directly affects inflammation [89].

In a study by Neuner *et al.* [66], it was shown that patients with high levels of IFN-γ had a four-year survival rate of 23% with a median survival of 13.3 months. Those with low IFN-γ had a lower four-year survival rate of 9.2% with a median survival of five months [66].

Table 1: Summary on inflammatory action of relevant cytokine inflammatory groups

Cytokine	Abbreviation	Pro- or anti- inflammatory cytokine
Interleukin-1 beta	IL-1ß	Pro-inflammatory [53; 54]
Interleukin-2	IL-2	Pro-inflammatory [23]
Interleukin-6	IL-6	Pro- and anti-inflammatory [49]
Interleukin-8	IL-8	Pro-inflammatory [75]
Interleukin-10	IL-10	Anti-inflammatory [77; 78]
Interleukin-12p70	IL-12p70	Pro-inflammatory [81]
Interleukin-17A	IL-17A	Pro- and anti-inflammatory [83;84]
Tumour necrosis factor alpha	TNF-α	Pro-inflammatory [44; 87]
Interferon-gamma	IFN-γ	Pro- and anti-inflammatory [89]

Data according to overall studies in patients and *in vivo*

Inflammatory markers relevant to NSCLC

To enable the prediction of high-risk lung cancer cases or early diagnosis, an immense research effort is understandably underway to identify biomarkers in blood, sputum, breath, airway epithelial cells and urine [91–93].

Several studies confirmed the results by Keeley *et al.* [22] that higher levels of select biomarkers were found in lung cancer patients, compared to control groups and higher levels of cytokines were found in future cancer diagnosed patients (Table 2). Barrera *et al.* [57] found that levels of IL-6, IL-8, IL-12p70, IL-17 and IFN-γ were significantly higher in patients with NSCLC than those of the controls. In another study by Trovo *et al.* [48], an increased level of interleukin 1 receptor antagonist (IL-1ra), IL-12, IL-17, IFN-γ and a decreased level of TNF-α were found in serum compared to the serum levels at baseline (Table 2).

Several studies confirm that inflammatory cytokines in cancer patients differ from that of the controls [22, 48, 57]. In NSCLC, the cytokines that differ between the controls and the patients are IL-1ß, IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-12p70, IL-17, TNF-α and IFN-γ (Table 2) [22, 57, 71, 72, 83].

In NSCLC patients, an upregulation of IL-1ß, IL-6, IL-8, IL-12p70, IL-17A and IFN-γ and a decrease in IL-2, IL-10 and TNF-α were found [57, 71, 72]. However, Keeley *et al.* [22] also indicated a significant increase in TNF-α ($p<0.01$) in patients, and Huang *et al.* [83] support this in a study where tumour tissue of NSCLC was compared to peritumour, and a significant increase ($p<0.05$) was also seen in these cancerous tissues (Table 2). In contrast, some cytokines did not differ from the control — these were IL-1ß [57], IFN-γ, IL-17A [71], TNF, IL-2, IL-4 [57; 71].

Lung adenocarcinoma with metastasis to lymph nodes has shown similar profiles of cytokines as that described by Barrera *et al.* [57], except for an increase IL-2 and IL-10 as well. IL-10 plays a definite role, as the addition thereof in NSCLC patients resulted in the secretion of IL-2 and IFN-γ (Table 2) [66].

The progression of cancer affects the cytokines even further as stage III and IV lung cancer's cytokine profile showed a significant elevation ($p<0.05$) when compared to stage 1 and 2 lung cancer [94]. This was confirmed by a significant decrease ($p<0.05$) in cytokines IL-1ß, IL-2, IL-8, IL-10, IFN-γ, and TNF-α in lung cancer patients who lived > 5 years compared to those with overall survival < 5 years [23]. Patients with lung cancer have shown a significant elevation in IL-6 and IL-8 ($p<0.001$), but when patients who have received treatment are compared to those without therapy, IL-8 levels significantly decreased ($p<0.001$) [74].

When comparing other cancers to lung cancer, the cytokine profiles differ. In head and neck cancers, an increase was seen in IL-12p70, but a decrease in IL-1ß, IFN-γ and TNF-α [22], and in patients with oesophageal cancer, a significant increase ($p<0.01$) was seen in IL-1ß, IL-12p70, IFN-γ and TNF-α [22].

Table 2: The effects of different cell lines, cancer types or cancer treatments on inflammatory cytokines. An increase in cytokines is indicated with (+), (++) indicates a statistically significant increase of $p < 0.05$, and (+++) indicates ($p < 0.01$). A decrease in cytokines is indicated (-), and significant decreases of $p < 0.05$ are signified by (- -), $p < 0.01$ is indicated by (- - -)

Subjects	IL-1ß	IL-2	IL-6	IL-8	IL-10	IL-12p70	IL-17A	IFN-γ	TNF-α	Other	References
Animal studies											
Mucosa of AKR1B8 mice	+ +		+ +					+ +			[95]
Large cell lung carcinoma cells in *in vivo* study in comparison to before cell inoculation				+							[96]
Patient related studies											
NSCLC patients	+	-	+ + +	+ + +	-	+ +	+ +	+ +	-		[57]
Lung cancer patients compared to benign lung diseases			+								[72]
Lung cancer compared to control groups			+ + +	+ + +							[74]
Lung cancer patients receiving treatment compared to those without treatment				- - -							[74]
Patients with head and neck cancer compared to control	-					+		-	-	IFN-α + IL17α + TNF-ß +	[22]
Patients with Lung cancer compared to control	+ + +					+ + +		+ + +	+ + +	IFN-α +++ IL17A +++	[22]
Patients with oesophageal cancer compared to control	+ + +					+ + +		+ + +	+ + +	IFN-α +++ IL17α +++	[22]

Table 2 (continued): The effects of different cell lines, cancer types or cancer treatments on inflammatory cytokines. An increase in cytokines is indicated with (+), (++) indicates a statistically significant increase of $p < 0.05$, and (+++) indicates ($p < 0.01$). A decrease in cytokines is indicated (-), and significant decreases of $p < 0.05$ are signified by (- -), $p < 0.01$ is indicated by (- - -)

Addition of IL-10 in NSCLC patients		-					-			[66]
Lung adenocarcinoma patients with lymph node metastasis in comparison with no node metastasis		+ +	+ +	+ +	+ +		+ +			[23]
Lung tumour in patients who survived > 5 years compared to those that survived < 5 years	- -	- -		- -	- -		- -	- -	IL1-α - - IL-12p35 - -	[23]
NSCLC patients		-	+++		-	+	+	-		[71]
Stage 3 and 4 freshly resected renal cell carcinoma tumours compared to Stage 1 and 2					++					[94]
Tumour tissue compared to peritumour tissue of NSCLC	++		++	++				++	IL-17RA +	[83]

Oxidative stress

In the maintenance of physiological processes, oxidative stress functions as a secondary messenger [9]. In many cancer-related processes, oxidative stress plays a vital role [10], where it is a contributory factor in the initiation, promotion and malignant conversion of cancer [31]. A change in the ration of ROS and antioxidants, caused by elevated levels of ROS, causes oxidative stress and damage [11–13; 97–98].

Depending on the imbalance, time and intensity, oxidant type and cell type, oxidative stress can influence the synthesis of antioxidant enzymes, thereby modulating signalling pathways, apoptosis, inflammation, cell proliferation and finally the oncogenic transformation [11, 15].

Oxidative stress does not only have negative results; for instance, after physical activity, the body produces free radicals (within a natural limit), which promote tissue growth. However, if this persists over the long term, it may cause oxidative stress resulting in cellular damage [99]. A delicate balance between ROS and antioxidants is therefore needed for proper cellular function, and the intracellular ROS levels should be maintained steadily to prevent cell damaging [11, 20].

Biomarkers of oxidative stress

Reactive oxygen species (ROS) are oxygen molecules that contains an unpaired electron, which makes it unstable. These molecules have short biological half-lives and target macromolecules, which can cause tissue or cell damage through damage to nucleic acids, lipids as well as proteins [16, 21, 97]. This may lead to DNA mutations, lipid peroxidation, and dysfunction of proteins [21]. Living cells produce ROS as a by-product of normal cellular metabolism and aerobic metabolism [12, 14, 15, 100] in mitochondria [101]. However, an irregular increase in ROS will lead to oxidative stress [15, 102].

Reactive oxygen species may cause redox modulation or redox signalling. When ROS acts as a second messenger and intervenes in responses that are necessary for proper cell functioning and survival, then it is identified as redox signalling. Redox modulation, on the other hand, is the process where ROS amends the functioning or activity of molecular targets that are redox-sensitive — metabolic enzymes or signalling proteins, which results in pathophysiological or physiological responses [14, 16, 17]. ROS also perpetuates inflammatory responses by activating cell signalling, amplifying the production and the release of pro-inflammatory cytokines [16].

Different diseases will release different amounts and types of ROS [18, 103]. In response to stress conditions or external stimuli, cells produce more ROS, forcing the living organism to have a series of mechanisms to adapt to this exposure to ROS [104]. These stimuli include growth factors,

cytokines, chemotherapeutics, environmental toxins and ultraviolet light [9, 15]. Cellular oxidative stress molecules are needed to oxidise proteins, lipids, as well as DNA, and for this ROS is a primary source; when ROS levels are excessively high, it will trigger feedback mechanisms such as oxidative stress, and promote the activation of necrosis, apoptosis and autophagy [14].

Oxidative stress can be determined by investigating the disproportion between ROS production and antioxidants [18]. In normal tissue and cell functioning, this balance is crucial [20]. In the majority of cancer types, elevated ROS levels have been detected and are responsible for the progression and development of tumours [20], and depending on the concentration, type and nature of the ROS, it may have several different functions in cancer [14, 17].

To maintain a high proliferation rate, cancer cells in comparison to normal cells have a higher rate of metabolism, which means they can maintain elevated ROS levels [14, 105]. Unlike other cancers that are primarily driven by lactate dehydrogenase (LDH) glycolysis (Warburg effect), lung cancer shows a high mitochondrial metabolism [106]. Both these before-mentioned pathways — LDH and mitochondrial metabolism — have increased ROS generation [107; 108].

ROS may also be toxic to cancer cells, and some cancer treatments were developed according to this process, where ROS is extensively increased to an extent where it reaches apoptosis [109, 110]. A high concentration of both ROS and antioxidant enzymes can lead to the proliferation and progression of tumours, but when the level of ROS is elevated and an imbalance occurs, ROS was shown to have anti-tumour effects [21].

Reactive oxygen species do not only play a role in cell damage, but also in intracellular signalling and regulation, and play a crucial physiological role in biological processes and may induce apoptosis [15, 97, 111, 112]. In the metabolism of certain drugs — such as doxorubicin, acetaminophen, atorvastatin, clozapine, tamoxifen and oestrogen — ROS is generated by phase 1 cytochrome P450 enzymes through its catalytic cycle [17, 111, 1130–118].

Antioxidants are natural or synthetic compounds that can neutralise excessive ROS levels in the body [97]. The antioxidant defences are crucial for the body, against its exposure to free radicals [9, 18, 19], and therefore protect cells from oxidative damage [19]. Endogenous antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), all of which protect vital biomolecules from oxidative damage [14, 16, 19].

The first major defence against oxidative stress is superoxide dismutase (SOD) [112]. This enzyme catalyses the reaction of free radical oxygen molecules into hydrogen peroxide (H_2O_2), which is a stable molecule [20, 112]. Catalase (CAT) and glutathione peroxidase (GPx) are abundantly

present in the body, especially in cells that detoxify toxic substances by using oxygen. The H_2O_2 that is produced in this detoxification is then converted into water and oxygen [112, 119].

In several studies that were conducted on different types of cancer, a decrease in oxidative enzymes' activity — SOD, CAT and GPx — was seen against an increase in oxidative stress and/or ROS [19, 120, 121]. Oxidative stress increased in correlation as the stage of lung cancer advanced, and proportionally the levels of antioxidant molecules decreased [10, 122].

Kaynar *et al.* [25] showed that, in patients with NSCLC, significant changes were indicated in the antioxidant defence system, where SOD and CAT increased, but lipid peroxidation also increased [25]. This means that the increase in oxidative stress initiated an increase in the defence system, but since the antioxidants' working was insufficient to counteract the oxidative stress, it led to cell damage [121]. Güner *et al.* [120] indicated a decreased level of antioxidant enzyme activity (SOD and CAT) compared to controls. Comparing lung cancer and control groups, ROS was increased, which may be due to the inadequate enzyme activity to reduce ROS [25, 120]. Huang *et al.* [19] reported that whenever cancer cells were exposed to anti-cancer compounds, the oxidative enzymes' activity increased.

Tumour proliferation is inhibited by antioxidants [123] and their main role is to protect against oxygen toxicity, which may lead to reactive oxygen metabolite levels being elevated [120]. There are many antioxidants (proteins, non-protein or enzymes anti-oxidants) that have anti-inflammatory activity [16].

Oxidative stress damage

When an excessive amount of ROS is present, which is beyond the capacity of the body's defence systems, it results in oxidative stress damage [9, 21, 124]. The cellular and DNA damage caused by secondary contributors, such as lipid peroxides, may initiate the malignant transformation of cancerous tissues [21, 95].

New covalent DNA- or protein adducts are formed when DNA or protein interacts with peroxides. This will lead to the dysfunction of proteins, DNA mutations and strand breaks, which, in turn, will lead to cell damage, genetic instability and the mutations of genes, and eventually to tumorigenic progression [95]. Several lipid peroxides are formed during lipid peroxidation, including α, ß-unsaturated carbonyls — malondialdehyde (MDA), acrolein, 4-hydroxynonenal (HNE), and crotonaldehyde [21, 125, 126]. Because MDA can arise from a few oxidative mechanisms, it is seen as a general indicator of lipid peroxidase, but not a specific marker [127].

These electrophilic carbonyl compounds, as a result of lipid peroxidation, increase the permeability and damage of membranes and lipid peroxide levels, by acting as a secondary pathogenic factor [21, 128–130]. Through secondary reactions of nucleophilic side chains and lipid peroxidation products — aldehydes— carbonyl (CO) groups may be introduced into protein [131]. Because of the electrophilic carbonyl properties, lipid peroxides are genotoxic [21, 95]. The majority of protein- and lipid damage caused by these lipid peroxides is irreparable [132]. Lipid peroxidation may also contribute to cell damage as a result of oxidising products being generated and the increase in oxidative stress levels, which eventually leads to inflammation [21, 130, 132].

Oxidative damage in cells is most likely to affect the proteins first, because they are more likely to be catalysts [133]. When the side chains of proteins are oxidised, carbonyl (CO) groups are produced [131]. Protein dysfunction due to carbonyls may impede the mitochondrial membrane potential — which can lead to apoptosis — and the mitochondrial respiratory chain reaction, resulting in elevated ROS production and its release in the cytosol [95]. Therefore, carbonyl lesions in inflammation may create a brutal cycle of oxidative stress, enhancing cell as well as tissue damage [5, 21]. The marker that is used the most for the modification of the oxidative status of proteins is protein carbonyl (PC) [134]. An increase in oxidative stress damage in cancer patients has been described, as shown in Table 3.

Table 3: The effects of different cell lines, cancer types or cancer treatments on oxidative stress biomarkers. An increase in biomarkers is indicated with (+), (++) indicates a statistically significant increase of $p < 0.05$, and (+++) indicates ($p < 0.01$). A decrease in biomarkers is indicated with (-), and significant decreases of $p < 0.05$ are signified by (- -), $p < 0.01$ is indicated with (- - -)

Subject group	ROS	SOD	CAT	MDA	PC	Other tests	References
***In vivo* and *in situ* studies**							
AKR1B8 mutant in C57BL/6 mice compared	+			+			[95]
NSCLC patients compared to control	+++	++	++	++		GSH +	[25]
SCLC patients compared to control	+++	+++	++	+++		GSH +	[25]
Lung cancer patients				+++			[24]
Oral cavity cancer patients		- - -	- - -	+++		GPx - GSH - Lipid hydro-peroxides +	[11]
Resected Lung cancerous tissue compared to normal tissue		- - -	- - -				[120]
***In vitro* studies**							
Most aggressive large cell carcinoma (HOP-92) compared to 6-fold less aggressive LCC cells (H-460.	++	+++	+++	++		GPx ++	[31]
Treatment of Glioma cells with copper-antineoplastic treatment	+ +	- - -	- - -				[121]
Untreated melanoma cell line (B16-F10)	+	-	-				[19]
Treatment of melanoma cells with Hinokitiol	-	+++	+++				[19]

Glutathione peroxidase (GPx); Glutathione (GSH); Large cell carcinoma (LCC)

Relationship between inflammatory cytokines and oxidative stress biomarkers

A relationship between oxidative stress and inflammatory markers has been found, where both affect and/or correlate with inflammation and the development and progression of differentiation of cells to develop cancer (Table 2 and Table 3).

Several attempts have been made to establish the connection between cytokines and oxidative stress, especially in cancer. This has proven to be difficult due to complicated pathways, which include inflammation. Different diseases seem to have different correlations between cytokines and oxidative stress markers [135–138]. Due to the paucity of direct relationships between cytokines and oxidative stress in lung cancer, the correlations of other diseases will also be discussed to elucidate an underlying trend.

In gestational diabetes, it was found that elevated MDA levels were associated with an increase in the total oxidative status, and an increase in TNF-α was associated with increased levels of MDA [136]. In diabetes, interleukin production is induced in response to elevated oxidative stress levels [139].

Early pathophysiological events of pancreatitis, an inflammatory process of the pancreas, showed that oxidative stress and cytokines play essential roles. It seems that nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein (MAP) kinase are responsible for the possible relation between cytokines and oxidative stress [135] (Figure 1). In pancreatic cancer, enzymatic pathways are enhanced by pro-inflammatory stimuli of IFN-γ, which lead to high amounts of extracellular ROS produced by pancreatic cells. This generation of ROS depends on the signalling pathways, of which NF- κB is one [138] (Figure 1).

Oxidative stress can be induced by inflammatory processes. The opposite effect is also possible, where oxidative stress is responsible for inducing inflammatory cytokines, by activating several pathways [140]. The most common pathway to be induced is the NF-κB [141, 142]. NF-κB is activated through secondary messengers — oxygen derivates — which lead to gene transcription and encoding of growth factors and cytokines [21, 143, 144]. Dysregulation of NF-κB signalling stimulates some pro-inflammatory markers (IL-1, IL-8, and TNF-α) and therefore promotes carcinogens and inflammation [21, 145] (Figure 1).

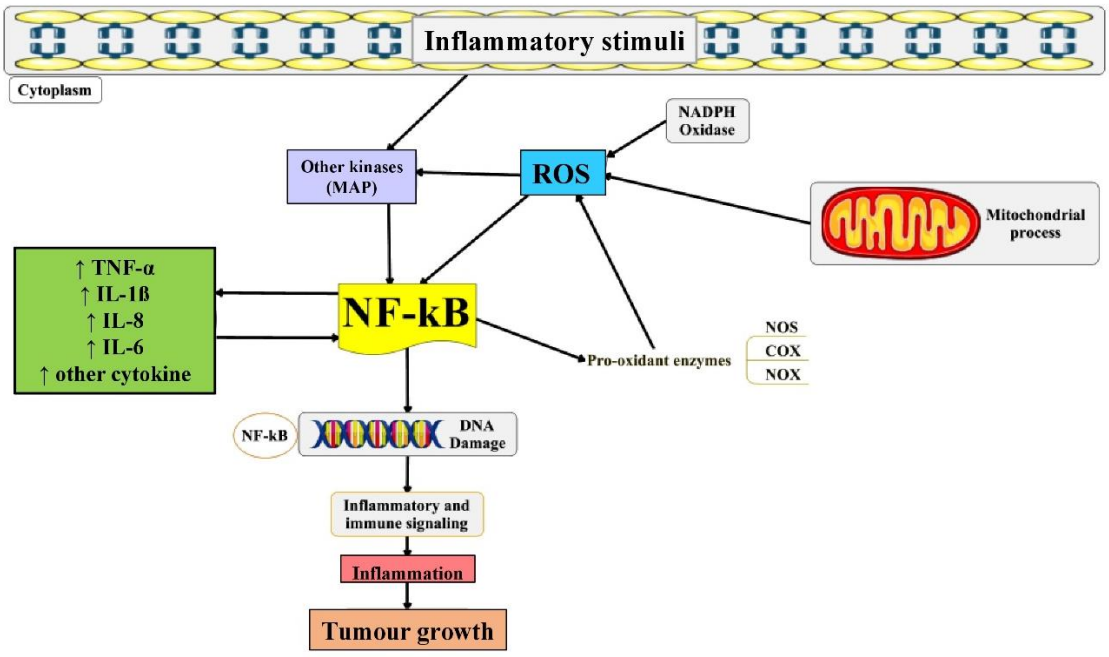

Figure 1: A summary of the role that nuclear factor kappa light chain enhancer of activated B cells (NF-κB) plays in ROS production, cytokine expression and inflammatory response. Figure generated from the combination of [135; 138; 147]

The activation of several stress kinases and factors, such as NF- κB, regulates genes for antioxidants as well as pro-inflammatory mediators (Figure 1). Through this activation, oxidative stress processes are suggested to have a vital role in inflammation [137]. No direct correlations could be drawn, but connections were rather more stimulus- and cell specific and not the rule [146].

In addition, ROS is induced by the normal mitochondrial process [147]. Co-stimulation of toll-like receptors (TLRs) induces higher levels of ROS production, externalising higher concentrations of endogenous adenosine triphosphate (ATP), which eventually leads to the elevated secretion of IL-1ß. The secretion of TNF-α results in regulation at a transcriptional level and ROS mediates it [148]. Antioxidants can restore production of IL-1RA and IL-6 [148], and therefore a direct link between oxidative stress and these cytokines can be deduced. In an oxidising microenvironment, the enhanced release of enzymes and molecules such as nitric oxide, superoxide, activation of NF-κB and the production of cytokines have been indicated [149] (Figure 1).

It is clear that the two pathophysiological processes — oxidative stress and inflammation — are connected. The one will induce the other; therefore, if oxidative stress appears first, it is likely that inflammation will follow; the opposite is also true, and these two linked processes will possibly develop in chronic inflammation, resulting in diseases such as cancer [140].

Inflammatory cytokines and elevated levels of ROS produced in cancerous tissue activate several signalling pathways, such as NF-κB, which are responsible for mediating cell proliferation,

differentiation and apoptosis of cells [150]. The carbonyl lesions and oxidative stress then cause DNA damage, which, in turn, plays a significant role in carcinogenic transformation [21].

Collins [151] stated that inflammatory cells were shown to release many different types of enzymes, chemical mediators and reactive species, which may eventually lead to oxidative stress, as well as cell and tissue damage. These inflammatory cells include activated phagocytic cells, such as macrophages and neutrophils [152].

Conclusion

As the search for new and improved diagnostics and treatment strategies continues, it is essential to understand the importance as well as the environment of cancer cells, with a focus on lung cancer as the most prevalent cancer [33, 39, 41].

It is well known that cancer is connected to inflammation, where inflammatory cells play an inevitable role in tumour growth, differentiation and migration [5]. With the information assembled in the investigation of the inflammatory markers relevant to NSCLC, it follows that a balance is needed between anti- and pro-inflammatory markers, since a dysregulation of the cytokines may result in cancer [3, 43].

Another important pathway in cancer is oxidative stress, which is normally a secondary messenger responsible for survival and apoptosis of cells [9]. An imbalance in ROS causes oxidative stress that can lead to oxidative stress damage and eventually result in cancer through damage to DNA, proteins and lipids [11–13, 97, 98].

From the literature, it can be concluded that in cancer patients and cancer models, an increase in pro-inflammatory cytokines generally means that there was an increase in ROS and oxidative stress damage markers (MDA and PC) and a decrease in antioxidants levels (SOD and CAT).

The exact relation between cytokines and oxidative stress markers in cancer has yet to be confirmed, as this entails a complicated network system and not a single responsible factor, enzyme or pathway. In other diseases, several possible connections were made: Because of elevated MDA levels as well as TNF-α in diabetes and in pancreatitis, a correlation was seen with elevated levels of IFN-γ, which enhanced ROS levels [134, 137, 138]. ROS levels depend on the NF- κB signalling pathway [137], which can possibly be a candidate in the connection between cytokines and oxidative stress markers in cancer [141]. Inflammation can be promoted by a dysregulation of NF- κB. This messenger can also influence both pro-inflammatory markers and antioxidants [136], which increases the possibility that NF- κB is one of the major correlating factors.

Conflict of interest

The authors have declared no conflict of interest.

Funding statements

This study was funded as part of completion of a M.Sc. degree in Pharmaceutical Sciences by the DST/NWU Preclinical Drug Development Platform.

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CHAPTER 3: RESEARCH ARTICLE

Chapter 3 was written as a research article, titled "*The effects of radiolabelled compounds on inflammatory cytokines and oxidative stress biomarkers"* for publication in the *Journal of Pharmacological and Toxicological Methods*. This chapter was written according to the Guide for Authors (https://www.elsevier.com/journals/journal-of-pharmacological-and-toxicological-methods/1056-8719/guide-for-authors) and is included in Appendix D. However, for ease of reading, the tables and figures are inserted at their appropriate positions in the manuscript. This manuscript will be submitted for publication.

THE EFFECTS OF RADIOLABELLED COMPOUNDS ON INFLAMMATORY CYTOKINES AND OXIDATIVE STRESS BIOMARKERS

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Abstract

The increase in cancer numbers annually confirms the urgency of the availability of improved cancer drugs. As further compromise of the immune system is not desirable, especially in unhealthy subjects, it is essential to look at secondary responses of drugs as well as the effect of these drugs on the immune system. The aim of this study was to establish the potential effects that newly developed radiolabelled compounds may have on inflammation and oxidative stress, by measuring the biomarkers of both conditions. Selected cytokines were analysed by means of flow cytometry using cytometric bead array and oxidative stress by enzymatic assays in 20 RNU female rats used to conduct this study. Methods: Cytokines were measured in the tissue and serum samples from xenografted RNU (RX) treated with two different radioactive compounds and control animals. Oxidative stress biomarkers were analysed using fluorospectrophotometry according to biomarker specifics. The result did not show a significant difference ($p < 0.1$) in the cytokines of the radiolabelled exposed group. A few significant ($p < 0.1$) changes were seen in the RX group compared to non-xenograft rats (RH). Even though the effect of radiolabelled compounds on cytokines and oxidative stress was not significant, a relationship between some markers was noted. Further investigations will help discover whether there are correlations between the biomarker reactions.

Keywords

Antioxidant, Biomarker, Cancer, Cytokines, Oxidative stress, Radiolabelled compounds, Xenograft

Abbreviations

A549 – adenocarcinomic human alveolar basal epithelial cells; CAT – catalase; Cu – copper; g – gravitational force; IL – interleukin; IFN – interferon; IVCs – Individual ventilated cages; TNF– tumour necrosis factor; MDA – malondialdehyde; Necsa – The South African Nuclear Energy Corporation; NSCLC – non-small-cell lung cancer; PC – protein carbonyl; Pd – palladium; PE – Phycoerythrin; RCF – relative centrifugal force; RCu – copper(II) chelate exposed animals; RDA – redundancy analysis; RH – Non-xenograft; untreated animals; RNU rats; baseline group – Rowett nude rats; ROS – reactive oxygen species; RPd – palladium(II) chelate exposed animals; RX – xenograft animals; SOD – superoxide dismutase; WHO – World Health Organization

Introduction

In 2018, cancer was the second leading cause of death globally, accounting for 9.6 million deaths (World Health Organization, 2018). This growing number of mortalities increases the urgency for new and improved diagnostic methods and effective treatments (Gilligan & Dwyer, 2017; Wang *et al.,* 2017). New and improved treatments for cancer are necessary to decrease the number of cancer-causing mortalities, and to alleviate the severe adverse effects of current drug regimens (Iqbal *et al.,* 2017).

Drugs containing atoms of radioactive elements can be classified as radiolabelled compounds, and are used for both diagnostic purposes and targeted therapy (Blower, 2015). They deliver ionising radiation to areas in the body with diseases, and may consist of macromolecules or even small organic molecules (Fichna & Janecka, 2003). The half-life of a radionuclide must be long enough in diagnostic imaging to enable the labelled compound's synthesis and to accumulate in target tissue, but to slow the clearance in the target tissue, while the non-target tissues and organs are cleared. Therefore, the half-life of a radiolabelled agent should be such that both these goals are met while at the same time limiting the radiation dose in the patients (Fichna & Janecka, 2003; Anderson & Welch, 1999). It is essential for radioisotope, chelated with diagnostic agents, to remain stable throughout the entire imaging, as data will not be useful if not stable (de Barros *et al.*, 2012).

Radiometals that are used for radiopharmaceuticals in positron emission tomography (PET) and single-photon emission computed tomography (SPECT) have half-lives that range from 10 minutes for ^{62}copper to several days for ^{67}gallium. The required half-life depends on the time required for the radiopharmaceutical to reach and localise in the tissue targeted. Tumour-targeting radiolabelled compounds take longer to reach the target than for example heart-targeting compounds. Additional important factors to consider in diagnostic agents are also the cost and availability of the agent (Anderson & Welch, 1999).

Radiolabelled compounds can be developed and synthesised according to pharmacokinetic requirements or demands (Fichna & Janecka, 2003). Although various elements are available to nuclear medicine, an element that is unique in the appropriate selection of radionuclides for diagnostic and targeted therapy is copper (Ikotun & Lapi, 2011; Boschi, Martini, Janevik-Ivanovska & Duatti, 2018).

It is believed that inflammation influences the development of cancer as well as the progression thereof (Brenner *et al.,* 2014). A correlation was found between radiation therapy and the changes in inflammatory markers (Bower *et al.*, 2009; Müller & Meineke, 2007). Dysregulation of inflammatory markers can be indicative of chronic inflammation, which may include cancer (Ghuman *et al.,* 2017; Keeley *et al.,* 2014; Trovo *et al.,* 2016). Tumour cell growth may also be

promoted by certain cytokines that are partly being produced in the tumours (Trovo *et al.,* 2016). In a study done by Keeley *et al.* (2014), the majority of the biomarkers tested were much higher in patients with lung cancer than in those of the controls. Therefore, the assumption is made that inflammation markers will differ between patients who have cancer and those without.

Various research efforts have been described related to biomarkers in blood, sputum, urine, breath and epithelial cells in the airway for the prediction of high-risk cases and early diagnosis (Spira *et al.,* 2007; Blomquist *et al.,* 2009). The improvements in endoscopic techniques and diagnostic imaging will also contribute to surgical refinements (Adams *et al.*, 2009).

In the regulation of physiological processes, such as survival, apoptosis and proliferative signalling pathways, oxidative stress plays an important role as a secondary messenger (Filaire *et al.*, 2013). In many cancer-related processes, there is oxidative stress involved (da Motta *et al.*, 2015). In lung cancer patients, antioxidants such as superoxide dismutase (SOD) and catalase activity (CAT) decreased. It may be due to an increase in the production of reactive oxygen species (ROS), which cannot not be detoxified due to possible inadequate enzyme activity (Güner *et al.*, 1996; Kaynar *et al.*, 2005).

This study aims to investigate the effects of treatment on two new radiolabelled compounds on the levels of selected cytokines and oxidative stress biomarkers in a xenograft rodent cancer model.

Materials and Methods

Materials

Cell culture

The A549 cell line (adenocarcinomic human alveolar basal epithelial cells) was obtained from the ATCC (Manassas, VA, United States of America). All tissue culture reagents and consumables were acquired from either Sigma-Aldrich (St Louis, MO, USA) (now Merck, Darmstadt, Germany) or Thermo Fisher Scientific (Waltham, MA, USA), unless otherwise stated, and were of analytical grade (>99%).

Radiolabelled compounds

The radiolabelled compounds were designed at the School of Chemistry, University of KwaZulu-Natal, South Africa, by Prof Akerman and his research group. The synthesis of these compounds was done at Necsa, South Africa. The chelate components of the compounds are novel and under development and will therefore not be disclosed in this manuscript.

The copper (II) chelate compound was administered intravenously through the tail vein. A dose of approximately 190 µg compound was administered to each rat (RCu). The palladium (II) chelate compound was administered to the rats (RPd) intravenously at a dose of approximately 60.9 µg per rat, through the tail vein.

Cytometric bead array

A Super-X Plex™ Cytokine flow cytometry assay kit and additional kits were bought from Antigenix America Inc. for cytokine analysis. The Super-X Plex™ flow cytometry assay kit (RX11122-C9) consisted of antibody conjugated beads, detection antibody biotin, and standards. The kit consisted of a 96-well filter plate, wash-, lysate assay- and reading buffer, streptavidin-PE conjugate and detection antibody diluent.

A rat serum/plasma diluent kit (DKM100), which contains mouse/rat serum, plasma assay buffer and mouse /rat serum, plasma standard diluent, as well as a cell lysate diluent kit (DKL100) that contained cell lysate assay buffer and cell lysate standard diluent, was bought additionally.

Biomarker analysis

Reagents used in the biomarker analysis were acquired from Merck (Darmstadt, Germany), unless otherwise stated. All reagents used were of analytical grade (>99%). Consumables were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Methods

Cell culturing method

The A549 NSCLC cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. The culture was kept in a humid environment of 5% carbon dioxide (CO_2) with the temperature at 37ºC. The cells were monitored daily for growth and confluence. Once a confluency of between 70 to 80% was reached, the cells were trypsinised, harvested and used to inoculate the animals in the *in vivo* study.

Animal selection, husbandry and group allocation

The Rowett nude (RNU) rats were used for this study, as they were first nominated as the best species on which to do the cancer study, as these rats are immunodeficient. Because of the immunodeficiency and the absence of T cells in these rats, the cytokine study is not as simple as would have been desired, but the majority of these inflammatory cytokines are nevertheless produced by B cells or natural killer (NK) cells, which the RNU rats have. Cytokines are produced and coordinated by several different cell types such as B cells, T cells, macrophages, neutrophils, etc. (Zhang & An, 2007). Therefore, we will still be able to use this species for the cytokine study as a cytokine level will be detected even in the absence of T cells.

Female RNU rats, between the ages of four and six weeks, were bred in-house at the DST/NWU Preclinical Drug Development Platform Vivarium, a good laboratory practice (GLP) certified and an AAALAC-accredited facility [NWU-00178-18-A5]. The rats received unique identification numbers and were housed in individual, ventilated cages (IVCs). Two to three rats of the same group were kept per single IVC, which was cleaned regularly, with food and water available *ad libitum*. The IVCs were kept in an environmentally controlled study room with 12h light/12h dark cycles. Routine monitoring was done daily. The animals were divided into four groups: the first group was the untreated non-xenograft control group (RH), which received no intervention (n=5), a cancer (xenograft) control (RX) (n=5), and two xenograft groups (n=5 each), exposed to the two distinct radiolabelled compounds (RCu and RPd).

Xenograft model and lifespan of rats

Fifteen animals (RX, RCu, and RPd) were inoculated with A549 cells at the age of four to six weeks. The xenografts were performed by harvesting viable cells re-suspended in a 1:1 Matrigel and plain Dulbecco's Modified Eagle Medium (DMEM) suspension. The animals were injected subcutaneously with 100 µL 1:1 Matrigel, plain DMEM suspension (1 000 000 viable cells), in the right hind leg, followed by close monitoring until the tumours have reached a volume size of 100 to 150 mm^3. The tumours were monitored and measured every second day. The tumours were limited to a maximum length of 40 mm (University of California San Diego, 2015). If the tumour volume exceeded this), the rats were euthanised to minimise distress and pain.

The tumour volume is calculated according to the following formula:

$$\text{Volume}\left(mm^3\right)=\frac{1}{2}\left(\text{length}\times\text{width}^2\right) \qquad \text{(Equation 1)}$$

Once the tumour had reached the desired size (100–150 mm^3), all the rats (RH, RX, RCu and RPd) were transported to the South African Nuclear Energy Corporation (Necsa). The rats were given seven days to acclimatise in IVCs, before the compounds were administered.

Groups RCu and RPd received copper (II) and palladium (II) chelate compounds, respectively. The rats were euthanised 24 hours post-administration of the compounds by an overdose of isoflurane followed by decapitation.

The animals from the control and baseline groups – RH and RX — were anaesthetised and euthanised on the same day as the radiolabelled exposed groups.

Animal euthanasia and sample collection

Once the animals were euthanised, blood was collected immediately, followed by centrifuging of the blood for five minutes at 3 000 RCF to obtain serum, followed by the dissection of animals. The serum, kidneys, liver and tumours — where applicable — were collected and radioactivity was determined. All samples were stored at -20ºC before and after analysis.

The samples remained stored at -20ºC until they were declared non-radioactive by the radiation officer at Necsa. These samples were then transported on ice to the laboratory at the DST/NWU PCDDP and stored at -80ºC until further use.

Sample preparation

Kidney, liver and tumour samples were mechanically homogenised in three different batches according to different buffers used in the various assays. The specific or relevant buffer of each assay was also added to the serum samples.

Cytokine analysis using flow cytometry

Selected cytokines in the tissue and serum were determined by means of a cytometric bead assay (Super-X Plex™ Cytokine Assay; Antigenix America Inc., USA). All reagents used were supplied with the kit. In short, 20 mg tissue was homogenised in 100 µL kit-specific homogenising buffer for the cytokines. Forty-five microlitres of capture bead suspension was added to the 96 well-filter plate, the solution was removed by vacuum, after which the beads were re-suspended with 30 µL assay buffer and 15 µL sample and the plate was sealed. After incubating for an hour at room temperature on a microtiter plate shaker, the plate was washed three times by removing the liquid by gentle vacuum and washing with the supplied wash buffer (100 µL each time). After washing, 25 µL biotinylated individualised cytokine antibody solution was added, sealed and incubated for 30 min. The plate was washed three times once more. Each well was filled with 25 µL Streptavidin-PE working solution. The plate was then sealed again and incubated for 20 min. After washing twice with 100 µL wash buffer, the supplied reading buffer (150 µL) was added to each well and agitated on a microtiter plate shaker. The plate was read on a Becton Dickenson (BD) Accuri® C6 flow cytometer. Prior to analysing the samples on the BD Accuri®, thresholds were set

for the standards to be applied on the samples — forward scatter was set to 350 000, and side scatter was set to 450 000. Gating was done on standards by using sizes of 4 µm and 5 µm, respectively, using the protocol to detect how groups should be labelled. The data were analysed using the FCAP Array™ software (version 3.0) from Beckton Dickenson. The assays were run in duplicate according to the manufacturers' assay protocol.

Biomarker analysis

All biomarker analysis results were reported relative to its protein content (mg/mL). Protein content was determined according to Bradford (1976). In short, each sample (5 µL) was added to 245 µL of Bradford's reagent and the absorbance was read at 590 nm on the SpectraMax® Paradigm® multimode detection platform. Samples were analysed in triplicate.

Biomarkers of oxidative stress

Reactive oxygen species (ROS)

The assay for ROS was conducted according to Socci *et al.* (1999) and modified by Flora *et al.* (2013). In the presence of ROS and esterase, the oxidative conversion of stable H_2DCFDA to 2, 7-dichlorofluorescein (DCF) (highly fluorescent) can be measured. A sample preparation was made using general homogenising buffer [0.1M potassium phosphate buffer, 1 nM EDTA, 1.15% potassium chloride (KCl), 20% glycerol, 0.1 nM phenyl methane sulphonyl fluoride] for a 10% (w/v) preparation. Homogenates and serum samples were further diluted to 0.25 % (w/v) with 40 mM Tris-HCl (pH 7.4). The assay was conducted on ice.

Each sample was divided into two fractions of 1 mL each. The first fraction served as a blank control – 40 µL methanol was added to each sample (to account for methanol's auto-fluorescence), and the second fraction 40 µL of 1.25 mM 2, 7-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Thermo Fisher Scientific (Waltham, MA, USA) dissolved in methanol was added for the estimation of ROS. All samples (fraction one and two) were incubated in a water bath at 37°C for 15 min. The fluorescence was read at 488 nm excitation and 525 nm emission on the SpectraMax® Paradigm® multimode detection platform. The samples were blanked by subtracting the blank from the loaded samples. The reading was expressed as relative fluorescence units (RFU) (Flora *et al.*, 2013; Socci *et al.*, 1999).

Superoxide dismutase (SOD)

The analysis for superoxide dismutase (SOD) was adapted from Del Maestro and McDonald (1985), as modified by Kim *et al.* (1995). The SOD assay is based on the principle of superoxide catalysing the pyrogallol autoxidation inhibition. Homogenising the tissues, 12 µL general homogenising buffer were used for 1.25 mg tissue.

A mixture of 50 mM Tris-buffer and 1 nM diethylene triamine penta-acetic acid (DTPA) (pH 8.2) was prepared in a 49-to-1 ratio. The DTPA/Tris buffer [1:49 v/v] (245 µL) was added to 4 µL sample and Tris-buffer blank in triplicate in a 96-well microtiter plate. The reactions started as soon as 4 µL pyrogallol (24 nM pyrogallol in 10 mM HCl) was added to each well. The kinetic reaction was recorded by measuring the absorbance every 30 seconds for five minutes at 560 nm on the SpectraMax® Paradigm® multimode detection platform, starting at time = 0 min for 11 intervals (five minutes) (Del Maestro & McDonald, 1985). The assay was performed on ice and in a dark room (pyrogallol is light sensitive). SOD was determined using the gradient of auto-oxidation of pyrogallol. SOD was expressed as ngSOD/mg protein (Del Maestro & McDonald, 1985).

Catalase activity (CAT)

The catalase (CAT) analysis was done according to Cohen *et al.* (1970). For this study, 3 mg tissue was homogenised in 30 µL general homogenising buffer. Homogenate and serum samples were centrifuged for 10 min at 4°C at 10 000 RCF.

The supernatant (10 µL) was added to a 96-well plate in triplicate. A volume of 93 µL hydrogen peroxide (H_2O_2) (6 mM) was added to each well, followed by mixing and three-minute incubation period. The reaction was stopped by adding 19 µL sulphuric acid (H_2SO_4), followed by immediately adding 130 µL of potassium permanganate ($KMnO_4$). A blank (10 µL phosphate buffer) and a standard (102 µL phosphate buffer, no H_2O_2) were also measured for each reading.

The absorbance of each sample was then read on the SpectraMax® Paradigm® multimode detection platform at 490 nm within 30 to 60 seconds. The catalase was calculated according to Cohen *et al.* (1970). CAT activity is expressed as µmol H_2O_2/min/mg protein. One unit of catalase activity represents the quantity of enzyme catalysing the decomposition of H_2O_2 (1µM) per minute (Li *et al.*, 2017).

Biomarkers of oxidative stress damage

Lipid peroxidation

The lipid peroxidation assay was done according to Ohkawa *et al.* (1979) as modified by Üner *et al.* (2005). To analyse the lipid peroxidation, the malondialdehyde content was measured. Twenty milligrams of tissue were homogenised in 100 µL 0.25 M sucrose buffer [250 nM sucrose, 25 mM tris-HCl] (pH 7.4) for the MDA assay. Homogenate and serum samples were centrifuged at 4°C for 10 min at 10 000 RCF and the supernatant used in the assay.

In a 2 mL microcentrifuge tube, 12.5 µL sample homogenate (supernatant) and 25 µL 8.1% sodium dodecyl sulphate (SDS) were added, followed by 187.5 µL 20% acetic acid (pH 3.5), 187.5 µL 0.8% thiobarbituric acid, and 87.5 µL deionised water. The samples were incubated at 95°C in a water bath for 30 min. Once cooled to room temperature, 125 µL deionised water and 625 µL butanol:pyridine solution (n-Butanol and pyridine [15:1]) were added. The samples were then vortexed and centrifuged for 10 min at 2 700 RCF at room temperature (23–25°C). The supernatant was read in triplicate at 532 nm (optical density) on the SpectraMax® Paradigm® multimode detection platform. Lipid peroxidation is reported as nmol/mg protein (Üner *et al.,* 2005).

Protein carbonyl formation

Protein carbonyl (PC) formation was analysed using the same method described by Floor and Wetzel (1998), as modified from Levine *et al.* (1990). In the PC assay, 500 µL general homogenising buffer was used to homogenise 50 mg tissue.

Homogenate and serum samples were centrifuged at 4°C at 10 500 RCF for 30 min. In a 2mL microcentrifuge tube, 500 µL of 2, 4-dinitrophenylhydrazine (DNPH) [10 mM in 2 M HCl] was added to 500 µL of the supernatant and incubated at room temperature for one hour and vortexed every 10 to 15 min. After the one-hour incubation period, 500 µL 6% trichloroacetic acid (TCA) was added to the samples to precipitate the proteins. The precipitate was then centrifuged at 10 000 RCF for three minutes at room temperature. The TCA was discarded carefully, and the protein pellet was washed three times by re-suspending it in 1 mL ethanol/ethyl ether (1:1). The sample was left to stand for 10 min before centrifuging and discarding the supernatant after each wash. After the final wash, 400 µL guanidine hydrochloride (6 M guanidine hydrochloride in 50% formic acid) was added to solubilise the proteins. The samples were left to stand for 15 min at 37°C, before centrifuging at 16 000 RCF for five min. The supernatant was then read in triplicate (100 µL each) on a 96-well plate at 366 nm on the SpectraMax® Paradigm® multimode detection platform. Protein carbonyls were reported as nmol carbonyls/mg protein (Floor & Wetzel, 1998).

Statistical analysis

The data obtained during this study were expressed as mean ± the standard error of the mean (SEM). The datasets were analysed using one-way ANOVA or the non-parametric Kruskal-Wallis test (depending on normality). Dunn's multiple comparison test was used to determine the significant differences between data. Significance was tested at a 90% and 95% confidence level. Statistical analysis was done on GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). Multivariate statistics were performed using Canoco 5 for Windows.

Results

The results of the cytokine analysis and the oxidative stress results are presented in Table 1 and Table 2, respectively. Of the cytokines, interleukin-1ß (IL-1ß) and interleukin-8 (IL-8) had the highest concentrations (1964 pg/mL and 3753 pg/mL, respectively) (Table 1). The organ group with the most detected cytokines (IL-1ß, IL-8, and IL-17A) was the kidneys, followed by tumour samples (Table 1). The lowest concentration of cytokines detected was for IL-2 and IL-10 (17.21 pg/mL and 20.45 pg/mL, respectively). The lowest concentrations of cytokines were also measured in the serum (Table 1).

Catalase activity was the greatest in the tumour samples (RX and RPd), whereas the least activity was measured in the serum (RH and RPd) (Table 2). RX serum and RPd tumours had the highest levels of SOD activity, whereas treatment with both RPd and RCu resulted in an inhibition of SOD (Table 2). The highest concentration of ROS was determined in xenograft control (RX) liver samples ($1.8x10^8$ RFU) and in the palladium exposure group's (RPd) kidneys ($1.67x10^8$ RFU) (Table 2). The lowest ROS concentrations were measured in the palladium- and copper exposure groups' serum and tumour samples, respectively (Table 2). Malondialdehyde content (MDA) was the highest in the RCu tumour samples (0.491 ng/mol), followed by the RPd kidney samples (0.241 ng/mol) (Table 2); the least amount of MDA quantified was in the tumour samples of RX and RPd (Table 2) (0.127 and 0.118 ng/mol, respectively). The PC levels in all samples were low with little variation between groups. (Table 2). Overall, the serum samples had the lowest levels of oxidative stress markers, whereas the tumours had the highest levels of oxidative stress markers.

Table 1: Inflammatory marker levels (pg/mL) in kidney, liver, serum and tumour samples of the Cu (II) chelate and Pd (II) chelate exposed groups, as well as a xenograft and healthy control. Results are indicated as a mean ± standard error of the mean (SEM)

		IL-1ß	IL-2	IL-8	IL-12p70	IL-17A	TNF-α	IL-6	IFN-γ	IL-10
RH	Kidney	1964±222.1*	210.8±24.31	880.3±128.4	522.7±75.33	2410±221.3*	385.1±45.78*	551.7±104.9	564±75.17*	174.9±20.41
	Liver	237.4±39.02	30.73±12.04*	22.06±9.407	33.98±5.247	299.1±53.48	51.78±9.296	30.53±5.523	42.8±7.119*	20.45±2.194*
	Serum	88.7±24.86	53.23±7.768	844.9±169.9*	208.8±48.89*	518±124.8	50.12±14.75	290.1±69.97	38.76±8.431	41.93±6.608
RX	Kidney	1129±133.3*	144.5±16.83	396.6±57.78$^{\#}$	366.7±23.2	1579±100.1*	236.3±14.08*	238±25.94	287.7±16.65*	115.6±5.449
	Liver	344.6±85.82	51.31±9.774*	49.35±15.64	87.07±20.65	290.6±76.21	70.35±9.868	60.1±18.27	95.15±18.57$^{\#}$*	71.37±13.52*
	Serum	48.33±8.178	17.21±6.785	355.6±89.68*	26.79±5.654*	347.5±47.86	63.86±23.19	107.5±25.85	27.52±6.002	26.93±4.864
	Tumour	176.5±63.86	62.52±50.6	3753±460.2$^{\#}$	166.5±91.42	290.4±26.95	60.04±25.12$^{\#}$	37.03±13.52	40.23±14.32	45.99±16.71
RCu	Kidney	1609±165.4†	130.8±21.08	1210±114.1$^{\#\dagger}$	473.7±38.03†	1579±135	317.7±32.09	366.9±54.36	345.8±40.8	156.1±12.98†
	Liver	235.4±32	26.51±4.23		30.8±3.22	284.4±34.62	47.52±11.24†	35.45±8.565	34.83±7.362$^{\#}$	33.14±2.536
	Serum	46.5±12.25	69.44±28.81	182.5±72.93	43.42±6.947	230.9±27.75	27.91±14.95	58.83±26.88	33.36±9.231	32.09±4.943
	Tumour	296.1±122.3	46.58±18.47	2680±316.8	185.2±79.12	222.6±58.99	186.2±43.36$^{\#}$	135.6±135.6	57±20.01	19.41±5.08
RPd	Kidney	829±162.7†	91.57±18.89	249.1±62.45†	267.9±62.1†	1011±258	198.2±43.54	179.8±46.63	228.4±49.38	70.48±15.48†
	Liver	456.6±168.4	56.15±8.264	109.9±59.92	59.54±11.26	481±86.98	105.5±14.35†	41.12±12.15	70.31±14.38	55.08±9.228
	Serum	33.3±10.4	48.09±18.9	334.9±112.3	29.77±6.09	293.2±79.51	71.86±8.438	172.8±72.57	29.26±7.507	28.01±5.921
	Tumour	319.3±111.2	26.84±15.13	256.7±13.75$^{\#}$	112±17.04	279.2±60.01	81.07±25.71	147.1±37.77	72.13±21.41	48.76±14.55

(*) Indicates significant results, compare to RH (healthy group)
($^{\#}$) showed significant results ($p<0.1$) of exposure groups compared to RX (xenograft animals)
(†) shows significant results ($p<0.1$) in the compound exposed groups
ND indicates non-detected results

Table 2: Oxidative stress marker levels in kidney, liver, serum and tumour samples of the palladium (II) chelate and copper (II) chelate exposed groups, as well as a xenograft and healthy control. Results are indicated as a mean ± standard error of the mean (SEM)

		ROS RFU	SOD ng SOD/mg protein	CAT µmolH2O2/min/mg protein	MDA nmol/mg protein	PC nmol carbonyls/mg protein
RH	Kidney	$8x10^7 \pm 1x10^7$	0.159±0.026	38.85± 6.114	0.169± 0.009	24.48± 3.58
	Liver	$9.25x10^7 \pm 1.55x10^7$	0.146±0.025	34.7± 9.88*	0.187± 0.018	12.49± 1.88
	Serum	$2.53x10^6 \pm 4.82x10^5$	0.231±0.035	14.26± 1.914	0.209± 0.053	5.726± 0.853
RX	Kidney	$1.3x10^8 \pm 1.52x10^7$	0.267±0.104	37.91± 8.9	0.167± 0.018	16.11± 2.643
	Liver	$1.8x10^8 \pm 3.98x10^7$	0.381±0.336	138.6± 12.86*#	0.150± 0.035	17.54± 6.626
	Serum	$3.61x10^6 \pm 1.05x10^6$	1.45±1.142	29.72± 7.245	0.187± 0.013	9.064± 1.81
	Tumour	$5.19x10^6 \pm 2.79x10^6$	0.405±0.117	142.9± 27.48	0.127± 0.015	28.16± 5.71
RCu	Kidney	$9.16x10^7 \pm 1.42x10^7$	0.345±0.032	55.24± 12.38	0.128± 0.023†	20.83± 1.535
	Liver	$8.64x10^7 \pm 3.54x10^7$	0.146±0.032	75.61± 8.689	0.163± 0.011	28.37± 2.957
	Serum	$3.90x10^6 \pm 7.59x10^5$	0.2359±0.037	21.97± 4.726	0.147± 0.020†	6.461± 1.11
	Tumour	$1.87x10^6 \pm 1.57x10^6$	0.228±0.093	133.6± 4.71	0.491± 0.172	23.98± 10.68
RPd	Kidney	$1.67x10^8 \pm 3.40x10^7$	0.213±0.044	63.31± 11.13	0.241± 0.037†	16.98± 4.724
	Liver	$1.61x10^8 \pm 3.34x10^7$	0.425±0.252	60.22± 11.05#	0.220± 0.024	17.73± 6.334
	Serum	$2.25x10^6 \pm 3.92x10^5$	0.193±0.054	13.35± 1.804	0.230± 0.020	8.982± 1.009
	Tumour	$8.02x10^6 \pm 3.93x10^6$	1.94±0.902	518.4± 227.4	0.118± 0.043†	12.31± 2.478

(*) Indicates significant results, compare to RH (healthy group)
(#) showed significant results ($p<0.1$) of exposure groups compared to RX (xenograft animals)
(†) shows significant results ($p<0.1$) in the compound exposed groups

Discussion

Table 3 presents the comparative increases or decreases of cytokines and biomarker levels between the four groups. All cytokines were decreased in the kidneys and serum, and increased in the liver. The cytokines detected in the liver and kidneys showed opposite trends after inoculation of the A549 cell line. The serum samples had similar reactions as the kidneys (Table 3).

The changes in cytokines in animals with a cancer xenograft are evident when comparing the untreated non-xenograft group — RH — with the xenograft control, RX. In the RX group's kidneys, IL-1ß, IL-17A, IFN-γ and TNF-α levels were decreased significantly ($p<0.1$) (Table 3). In the livers of RX animals, IL-2, IL-10, and IFN-γ were increased significantly ($p<0.1$). The serum cytokine levels of IL-8 and IL-12p70 were significantly ($p<0.1$) decreased in the RX group compared to RH. In a study with NSCLC patients, different results were shown, where compared to controls showed a significant increase ($p<0.05$) in serum levels of IL-12, IL-17, and IFN-γ (Trovo *et al.*, 2016).

Exposure of the animals in the radiolabelled compounds (RCu and RPd) exposed or treated groups resulted in different effects on the cytokines relative to the xenograft model (RX). The copper (II) chelate exposure resulted in an overall increase in cytokines in the kidneys, where IL-8 was significantly increased ($p<0.05$) (Table 3). In the liver of RCu exposed groups, cytokines were decreased, with IFN-γ levels decreased significantly ($p<0.1$).

Serum and tumour samples of the animals of the RCu group showed no visible trend in the cytokine changes relative to the RX control. Only TNF-α measured in the tumours of the RCu group had a significant increase ($p<0.05$) (Table 3).

Palladium (II) chelate treatment resulted in a decrease in all cytokines in the kidneys. In the liver, an overall increase was noted, except for IL-12p70, IL-6, IFN-γ and IL-10 (Table 3). The serum also showed an overall increase, except for IL-1β, IL-8 and IL-17A. The decrease in IL-8 levels in the tumour was the only significant change ($p<0.05$) in all of the palladium exposed (RPd) samples compared to RX (Table 1). With the exception of copper increasing IL-8 in the kidneys, increasing TNF-α in the tumour, decreasing IFN-γ in the liver, and palladium decreasing IL-8 in tumour, no other significant data were found in the radiolabelled exposed groups compared to the RX group.

Table 3: The effect of xenograft and compound exposure on inflammatory markers and oxidative stress markers in different samples (p<0.1)

		Pro-inflammatory cytokines						Pro- and anti-inflammatory cytokines		Anti-inflammatory cytokine	Oxidative stress markers			Oxidative stress damage biomarkers	
		IL-1ß	**IL-2**	**IL-8**	**IL-12p70**	**IL-17A**	**TNF-α**	**IL-6**	**IFN-γ**	**IL-10**	**ROS**	**SOD**	**CAT**	**MDA**	**PC**
RX effect compared to RH	Kidneys	↓↓	↓	↓	↓	↓↓	↓↓	↓	↓↓	↓	↑	↑	↓	↓	↓
	Liver	↑	↑↑	↑	↑	↑	↑	↑	↑↑	↑↑	↑	↓	↑↑	↓	↑
	Serum	↓	↓	↓↓	↓↓	↓	↑	↓	↓	↓	↑	↑	↑	↓	↑
RCu compared to RX	Kidneys	↑	↓	↑↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	↓	↑
	Liver	↓	↓	-	↓	↓	↓	↓	↓↓	↓	↓	↓	↓	↑	↑
	Serum	↓	↑	↓	↑	↓	↓	↓	↑	↑	↑	↓	↓	↓	↓
	Tumour	↑	↓	↓	↑	↓	↑↑	↑	↑	↓	↓	↓	↓	↑	↓
RPd compared to RX	Kidneys	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↑	↑	↑
	Liver	↑	↑	↑	↓	↑	↑	↓	↓	↓	↓	↑	↓↓	↑	↑
	Serum	↓	↑	↓	↑	↓	↑	↑	↑	↑	↓	↓	↓	↑	↓
	Tumour	↑	↓	↓↓	↓	↓	↑	↑	↑	↑	↑	↑	↑	↓	↓
RPd compared to RCu	Kidneys	↓↓	↓	↓↓	↓↓	↓	↓	↓	↓	↓↓	↑	↓	↑	↑↑	↓
	Liver	↑	↑	-	↑	↑	↑↑	↑	↑	↑	↑	↑	↓	↑	↓
	Serum	↓	↓	↑	↓	↑	↑	↑	↓	↓	↓	↓	↓	↑↑	↑
	Tumour	↑	↓	↓	↓	↑	↓	↑	↑	↑	↑	↑	↑	↓	↓

In a comparative percentage composition of the different cytokines measured in the different tissue and serum samples for every animal group, a better understanding of the ratio of cytokines can be seen (Figure 1). A comparison of the liver and kidney composition of cytokines showed that their compositions are similar to each other — IL-10 being the cytokine detected the least. In the serum samples, an overall slight increase in IL-8 and a decrease in IL-1β are present. The tumour tissue shows a significant difference in cytokine composition compared to the other samples. Interleukin 8 showed a significant increase in RX and in RCu, but in the RPd it was decreased. The cytokines — IFN-γ and IL-17A — showed a decrease in the percentage composition in all groups of the tumour tissue. In the RPd group of tumour tissue, IL-1β was much higher than RX and RCu (Figure 1).

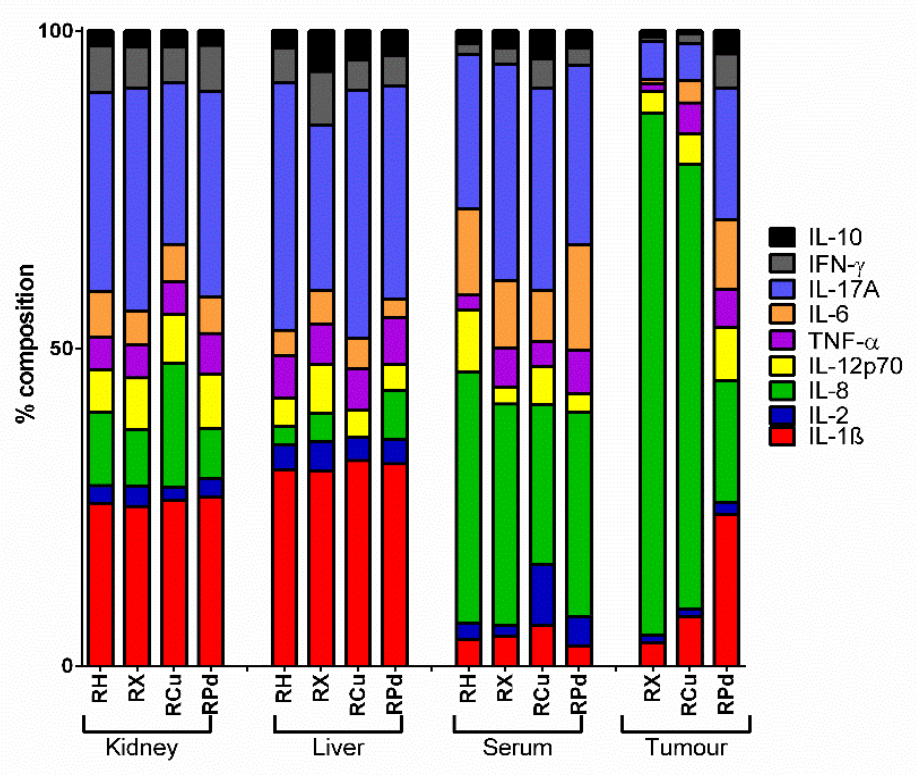

Figure 1: The percentage (%) composition of the cytokines measured in different tissue or serum samples in each group of RNU rats

A principal component analysis (PCA) was performed on the cytokine levels in the tissue samples from all groups. It is based on a linear response model that explains the variance in the dataset. Results are interpreted according to Šmilauer and Leps (2014). Correlation between variables is indicated by the angle between their vector lines; the closer to 0° the stronger the correlation.

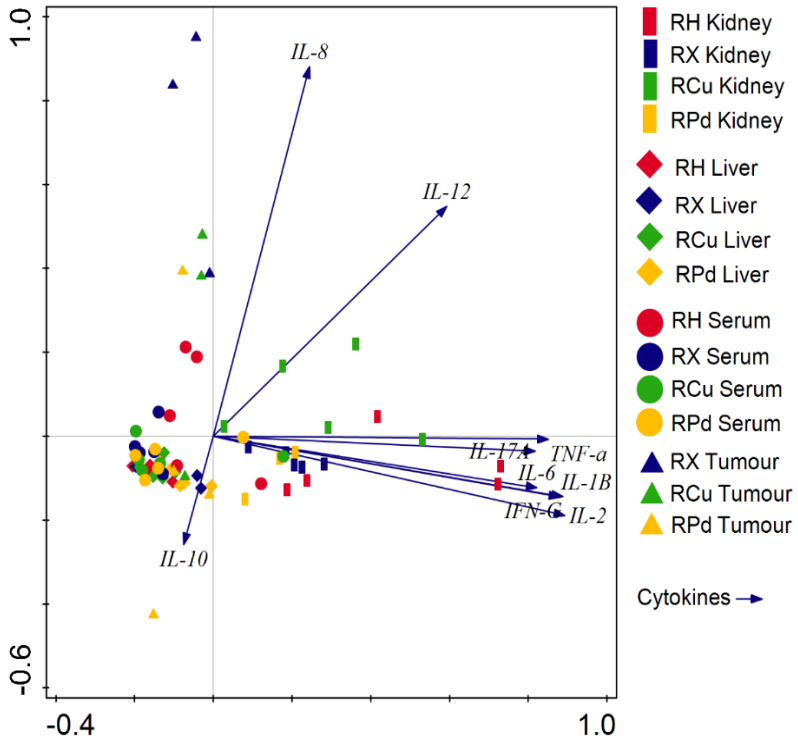

Figure 2: PCA biplot of cytokine levels in the kidney, liver, serum and tumour (where applicable) from the experimental groups and control. The ordination explains 67.58% of the variance in the data. (IFN-G = IFN-γ)

Factor 1 (53.85%) distinguishes between the samples and groups that had the higher cytokine levels (positive right side) and those who had low to no cytokines detected (negative left side). Factor 2 (13.75%) divides between anti-inflammatory (negative bottom) and pro-inflammatory (positive top) cytokine reactions. This is also confirmed with the strong negative correlation between IL-10 and IL-8 (vector lines 180° to each other). There is a moderate positive correlation between pro-inflammatory IL-8 and IL-12p70 (<45° between vector lines) (Figure 2). The remaining cytokines (IL1ß, IL-2, IL-6, IL17A, IFN-gamma, and TNF-α) have a strong positive correlation to each other, and no correlation to either IL-8 or IL-10 (90° between vector lines).

Interleukin-2 is required to produce TNF-α and IFN-γ (Capobianco, Cassiano & Antônia, 2016); it is then expected that TNF-α and IFN-γ will respond to changes in IL-2. This is confirmed in the above results (Table 1) and supported by the PCA (Figure 2).

Dinarello and Netea (2014) stated that IL-1 and TNF-α may possibly work together; this is also reflected in our results (Table 1 & 3). The results of IL-6 and IL-10 are supported by Yasukawa *et al.* (2003), who showed the prolonged action of IL-6 had the same anti-inflammatory response than that of IL-10. Interleukin-6 was decreased in the RX group when compared to the non-treated control (RH) in kidney and serum samples. These results are in line with Yanagawa *et al.* (1995)

and Pine *et al.* (2011), who found that patients diagnosed with lung cancer serum had higher levels of IL-6 (in serum).

Levels of IL-8 in this study were in contrast to the findings of Seike *et al.* (2007) and Pine *et al.* (2011), who reported significantly elevated IL-8 levels in tumour tissue compared to non-cancerous tissue. In the current study, IL-8 was only increased in the liver of the xenograft control (RX). Nanchahal, Taylor, Williams and Feldmann (2014) showed that when TNF-α is reduced, IL-6 and IL-1ß will probably also decrease. This trend was also noted in results (Table 1 & 3).

The kidney samples showed a significant decrease ($p<0.05$) in IFN-γ in the RX compared to RH. This cytokine can inhibit IL-8 and IL-1 (Mühl & Pfeilschifter, 2003), but this is in contrast with the findings of this study — as the increase in IFN-γ levels did not correspond with a decrease in IL-1 and IL-8 (Table 3). These differences may be due to species-specific immunity (Bosschem *et al.,* 2017; Seabrook *et al.*, 2004).

The intracellular ROS was increased in the RX group's kidneys, liver and serum, relative to the healthy untreated control (RH). As expected, as ROS increased, the anti-oxidant enzymes also increased (Table 3). The increase in ROS in the cancer model supports previous studies that also showed a higher level of ROS in the progression and development of cancerous tissue (Liou & Storz, 2010). No statistical levels of ROS were measured in the treatment groups (RCu and RPd), indicating that these compounds do not have an innate oxidative effect. Similarly, no statistical changes in the antioxidant enzymes were noted, with the exception of CAT in the livers of the xenograft control (RX) and palladium treatment group (RPd). The statistical increase ($p<0.1$) in the CAT levels of the RX livers coincides with the ROS present (Table 3). The same reasoning applies to the statistical decrease of CAT in the RPd group — and for the RCu group – i.e. low ROS concentrations result in a low CAT activity. The lack of PC and MDA (Table 2) also shows that the treatments had no oxidative stress effect on the animals. According to the literature (Kaynar *et al.*, 2005, Huang *et al.*, 2015), oxidative stress damage has been found to be associated with cancer. A PCA was performed to determine the variance in the data between the oxidative stress biomarkers in all the groups (Figure 3).

The PCA graph for the effects on oxidative stress biomarkers represents 47.85% of all results. Reactive oxygen species (ROS) have a weak negative correlation to SOD, and a positive correlation to CAT, which may indicate that the more ROS produced, the more CAT is released in defence to the possible oxidative stress. ROS also has a positive correlation with PC, although weak, which indicates that the higher levels of ROS induce protein damage (Figure 3). From this, the assumption can be made that the antioxidants have a negative correlation to oxidative stress damage, meaning that the higher the antioxidant levels are, the less damage will occur.

Factor 1 (25%) indicates the serum samples and SOD biomarkers on the right (positive side) and the other tissue samples – kidneys and liver – on the left (negative side). The SOD levels in serum samples were the highest detected SOD levels (Table 2 and Figure 3). Factor 2 (22.85%) divides the oxidative stress damage – PC and MDA (negative bottom) and CAT (positive top). It therefore explains that if CAT is absent, damage will occur.

The ordinations indicated a negative correlation between SOD and PC, as well as between MDA and CAT. This is expected since with an increase in antioxidants, there will be a decrease in ROS and similar oxidative compounds, resulting in less oxidative stress damage.

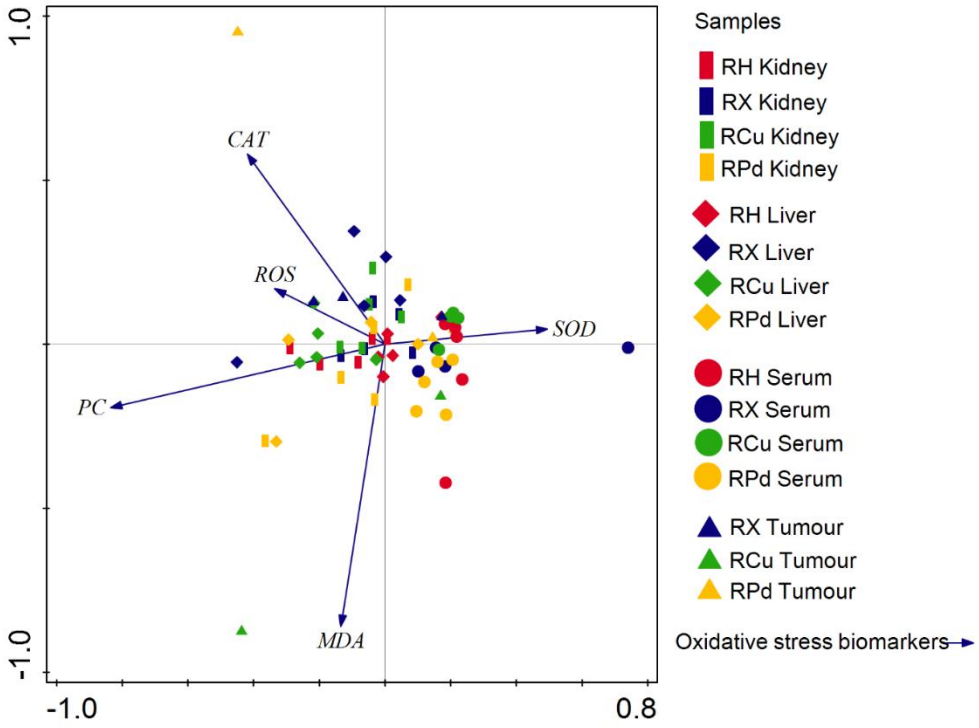

Figure 3: PCA graph to show the effects the oxidative stress biomarkers have on each other

The comparison between the two compound groups — RPd and RCu — does not give an indication of the effects of these compounds on the cancer, but the comparison between these two groups gives an idea of the difference in effects on the cytokines and oxidative stress markers (Table 3). In the kidneys of the RPd animals, there were lower levels of cytokines, and in the liver, an increase compared to RCu. ROS, CAT and MDA levels were statistically higher ($p<0.1$) in the RPd group's kidneys compared to RCu. In the liver, ROS, SOD and MDA were elevated compared to RCu. Serum samples showed a significant increase in only the damage biomarkers — PC and MDA ($p<0.1$).

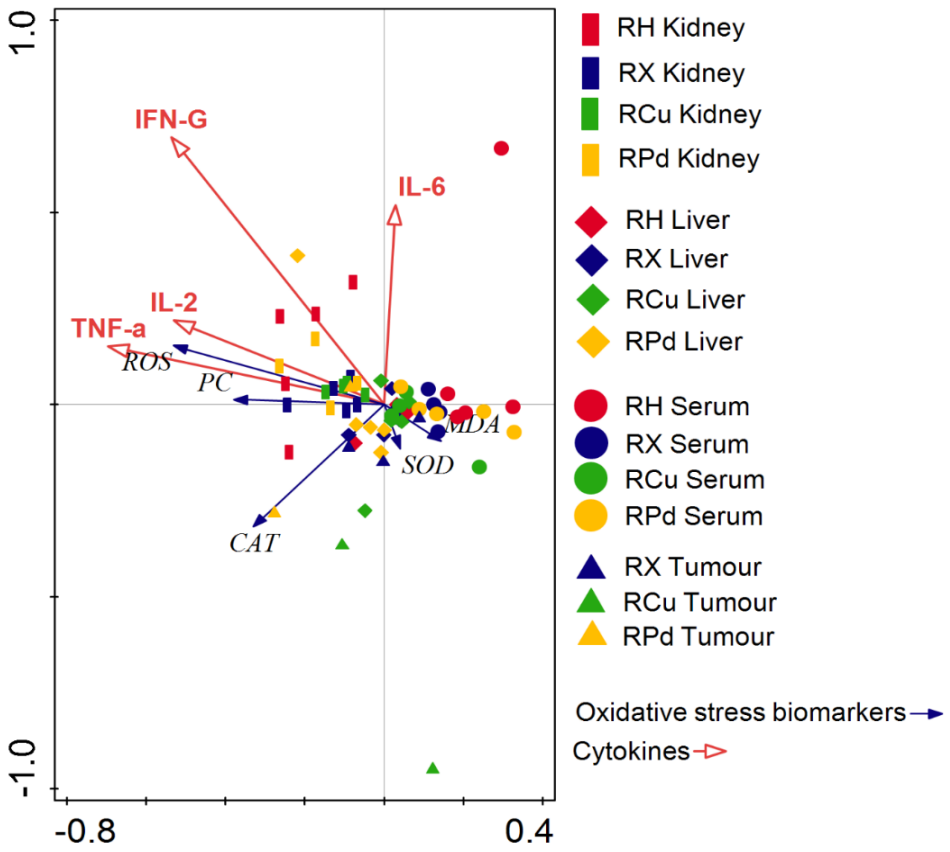

Figure 4: Forward selected redundancy analysis (RDA) triplot of cytokines and oxidative stress markers from the experimental groups and control. The ordination explains 14.7% of the variance in the data. (IFN-G = IFN-γ)

To determine similarities or differences between the cytokines and oxidative stress biomarker datasets, a redundancy analysis (RDA) was done (Figure 4). Strong co-linearity of the cytokine data meant that the forward selection option in the RDA was used. This reduces overestimation in explained variance. Only 14.7% of the variance in data is explained by RDA. Factor 1 (X-axis) showed 11.14% of the variance and axis 2 (Y-axis) only 2.9%.

The difference in concentrations is represented by axis 1, where serum is situated on the right and the tissue samples, which are responsible for the responses, are on the left. Axis 2 explains the least variance. This axis is responsible for the differentiation between the antioxidants compared to the pro-inflammatory cytokines. The low variance explained by the RDA reveals that there are other variables that were not measured or included in this study, which have an effect on both the cytokines and biomarkers. However, those contributions made by variables IL-2, IL-6, and IFN-γ, were significant ($p<0.05$). There is a strong positive correlation between the ROS, PC, IL-2 and TNF-α (Figure 3). A weak positive correlation is present between IFN-γ and IL-6 (both are anti- and pro-inflammatory markers), but PC and ROS have a positive correlation to each other (Figure 4); this was also found in the PCA (Figure 3).

Overall, a higher concentration of cytokines was seen in the kidneys and especially in the RCu groups, specifically IL-1ß, IL-8, and IL-17A. These three cytokines were also the dominant in the cytokine composition in the kidneys and the liver (Figure 1). The lowest concentrations of cytokines were detected in the serum samples. Interleukin-2 and IL-10 were the lowest concentrations overall (Table 1 and Figure 1). The palladium treatment was responsible for a decrease in cytokines (Table1; Table 3; Figure 2). For the oxidative stress markers, the serum had the lowest levels, and the tumour samples the highest.

One explanation as to why the kidney samples had the highest concentrations of cytokines can be that both the copper- and palladium-labelled compounds are metabolised by the renal system. The increased accumulation due to excretion can lead to an increased exposure of the kidneys to these compounds and so a greater response is elicited. Bio-distribution data should clarify the difference found between tissue samples. It will then be possible to conclude what effect these compounds will have on the cytokines and the oxidative stress biomarkers in the specific metabolic system.

Conclusion

The levels of oxidative stress biomarkers and cytokine concentrations of the copper compound treated group (RCu) were the closest to the healthy group. The palladium compound was responsible for the biggest changes seen in the cytokines and oxidative stress biomarkers.

The measurement of cytokines is potentially a valuable preclinical drug development screening tool. As shown in the current study, a change in the inflammatory markers of physiologically important systems, such as renal and hepatic, is detectable after treatment with or exposure to compounds. With the knowledge that there is a potential effect on the immune responses, the safety of the drug under development can be further scrutinised, by combining these screening tools with other specialised drug safety testing assays.

Ethical considerations

This study was approved by the NWU-AnimCareREC (Animal research ethics committee) [NWU-00178-18-A5]. The animal study was designed according to the three Rs — replace, reduce and refine. Parts of tissues from animals exposed to radiolabelled compounds used in this study were also used in another study regarding compound development. In this manner, the numbers of animals used were reduced.

Declaration of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank Dr Cathryn Driver, Dr Ambrose Okem, Dr Nico Minnaar, Mr Cor Bester and Mr Kobus Venter for assistance in the animal study. We thank Mr Emile Jansen van Rensburg for technical assistance during the flow cytometry analysis. Lastly, we want to thank Prof Faans Steyn from the NWU Statistical Consultation Services.

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CHAPTER 4: FINAL CONCLUSIONS AND RECOMMENDATIONS

Despite the fact that cancer remains a serious global health concern, few new drugs without serious adverse events or negative effects on the immune systems are becoming available. This motivates further preclinical investigations into the use of novel compounds for treatment or tools for early diagnosis of cancer (Kenny & Marmion, 2019). It may also mean that the array of preclinical studies needs to be expanded to include other markers, such as oxidative stress biomarkers for secondary responses, or markers of the effect of the drug on the immune system – such as cytokines (Tarrant, 2010).

As part of the development of radiolabelled compounds for either the diagnosis or treatment of cancer, this study aimed to look deeper into an analysis of the effect of these compounds on the immune system, as well as on the secondary responses in oxidative stress biomarkers. Comparing these levels of markers to absolute control animals as well as xenograft control, it was possible to evaluate these levels.

In this chapter, the findings from previous chapters are summarised and final conclusions drawn. Recommendations will also be made for similar future studies.

4.1 Final discussion and conclusions

During this study it was pertinent to understand the effect that the xenograft had on the target markers compared to an absolute control (no intervention), therefore a xenograft control was included. The changes in cytokines in animals with a cancer xenograft were evident when comparing the untreated non-xenografted group (RH) with the xenografted control (RX). In the RX group's kidneys, interferon-γ (IFN-γ), interleukin-6 (IL-6) and interleukin-8 (IL-8) were decreased two-fold compared to RH (Figure 1a); SOD was increased six-fold when comparing the RX animal group to the absolute control — RH. In the liver, several cytokines (IL-6, IL-8, IL-10, IL-12p70 and IFN-γ) were increased by two- to three-fold (Figure 1b). In the liver of RX animals, reactive oxygen species (ROS) and the antioxidants — superoxide dismutase (SOD) and catalase (CAT) — were increased two- to four-fold (Figure 1b). An increase in the antioxidants — SOD and CAT —– was seen (6- and 2-fold, respectively) in the serum of RX compared to RH animals (Figure 1c), while a decrease in the cytokine levels was seen. Interleukin-2, IL-6 and IL-8 were decreased by two- to three-fold, and IL-12p70 was decreased eight-fold (Figure 1c).

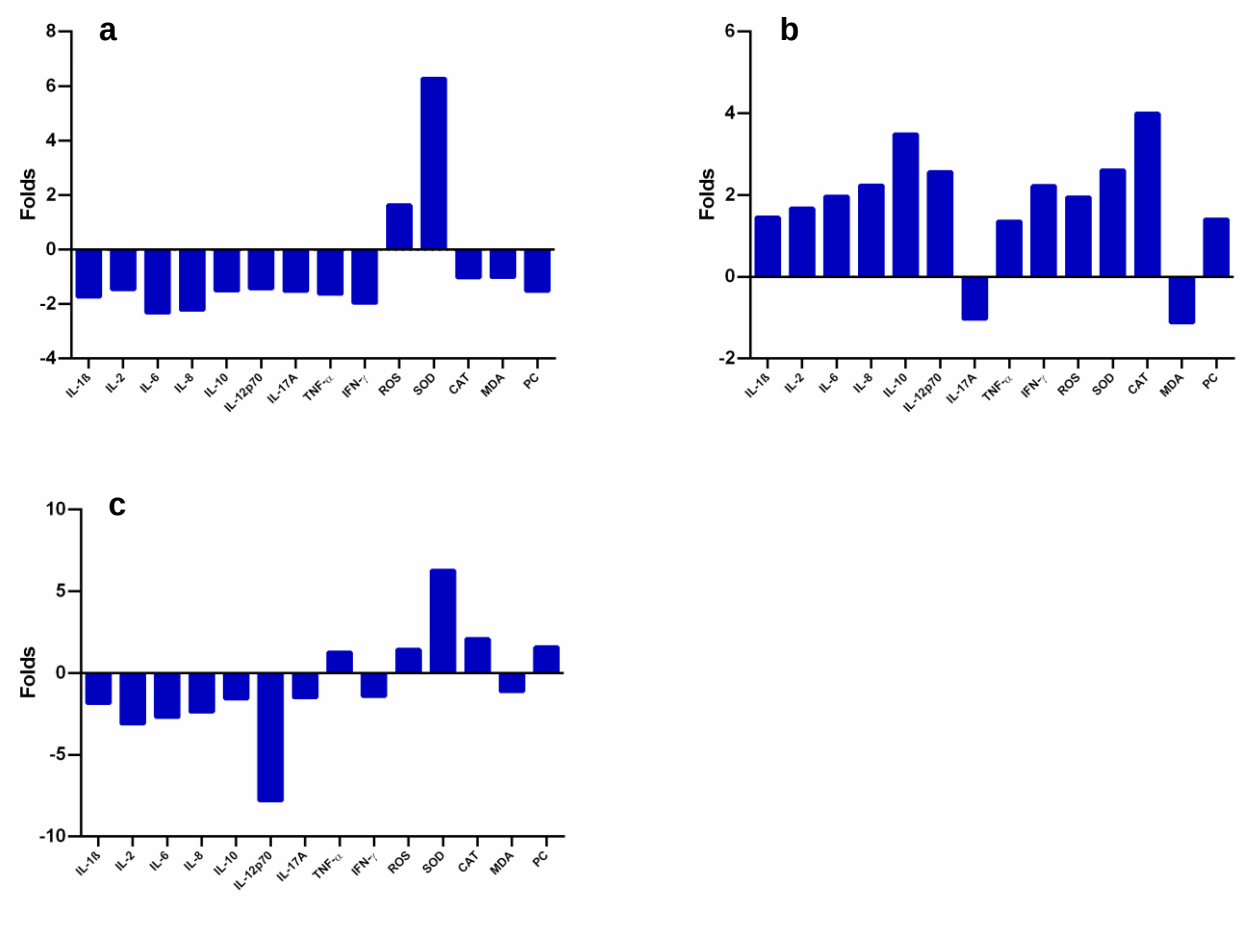

Figure 1: The comparison of cytokine levels as well as oxidative stress biomarkers (fold) between the RX and RH animal groups in the kidneys (1a), the liver (1b) and the serum (1c)

Exposure to the two radiolabelled compounds — copper (II) treated (RCu) and palladium (II) treated (RPd) compared to the RX group led to differences in both the cytokine and oxidative stress biomarker levels. In the kidneys of both RCu and RPd exposed animals, the changes were not significant, but in the kidneys (Figure 2a), the RCu group compared to RX group showed an average increase in the majority of levels, and the RPd compared to the RX group showed a decrease in the majority of levels. In the liver, the opposite was observed, with a decrease in the majority of levels after RCu exposure and an increase after RPd exposure when compared to the control RX group (Figure 2b). These findings may be explained when biodistribution data, which form part of a different study (Chapter 1: Study dependancy), become available. It is important to note that even though these changes were not significant between the treatment groups (Figure 2), that this is a meaningful result[1] — the fact that these responses were opposite may indicte that there is clinical relevance, which should be further investigated.

[1]Scientists are moving towards dismissing the misuse of p-values as an arbitrary threshold of statistical difference. Please refer to *Nature* (2019) 567, 305–307; *The American Statistician*, (2019) 73, sup1,1—19, doi: 10.1080/00031305.2019.1583913

In the liver of the RCu group, a two- to three-fold decrease was seen in IL-2, IL-10, IL-12p70 and IFN-γ. A similar decrease was aslo noticed in the ROS and SOD levels compared to the control xenograft animals RX (Figure 2b). The biomarker levels of the RPd group compared to the RX group showed a two-fold increase in IL-8, but a two-fold decrease in CAT levels.

In serum samples of the RCu group, an elevation of four-fold was seen in IL-2, and a decrease of two-fold was seen in IL-8 and TNF-α. The antoioxidant — SOD — of the RCu exposed animals showed a decrease of six times compared to the SOD of the RX animals (Figure 2c). A similar decrease in SOD was seen in the RPd group compared to the RX group, with an eight-fold decrease in addition to a two-fold decrease in CAT. The only difference in cytokine levels in the serum samples of the RPd group in comparison to the RX group, worth mentioning, was the three-fold increase in IL-2 (Figure 2c).

In the tumour samples of the RCu group compared to RX, a three- to four-fold increase in TNF-α and IL-6 was seen, as well as a two-fold decrease in IL-10. The reactive oxygen species (ROS) decreased three-fold, but the lipid damage (malondialdehyde (MDA)) increased four-fold (Figure 2d) in the RCu group. The RPd group showed a three- to five-fold increase in SOD and CAT (antioxidants), and a two-fold decrease in protein damage (protein carbonyl (PC)) compared to the RX group (Figure 2d). This group also showed an increase of four-fold in IL-6, but a two-fold decrease in IL-2 and a 15-fold decrease in IL-8 (Figure 2d).

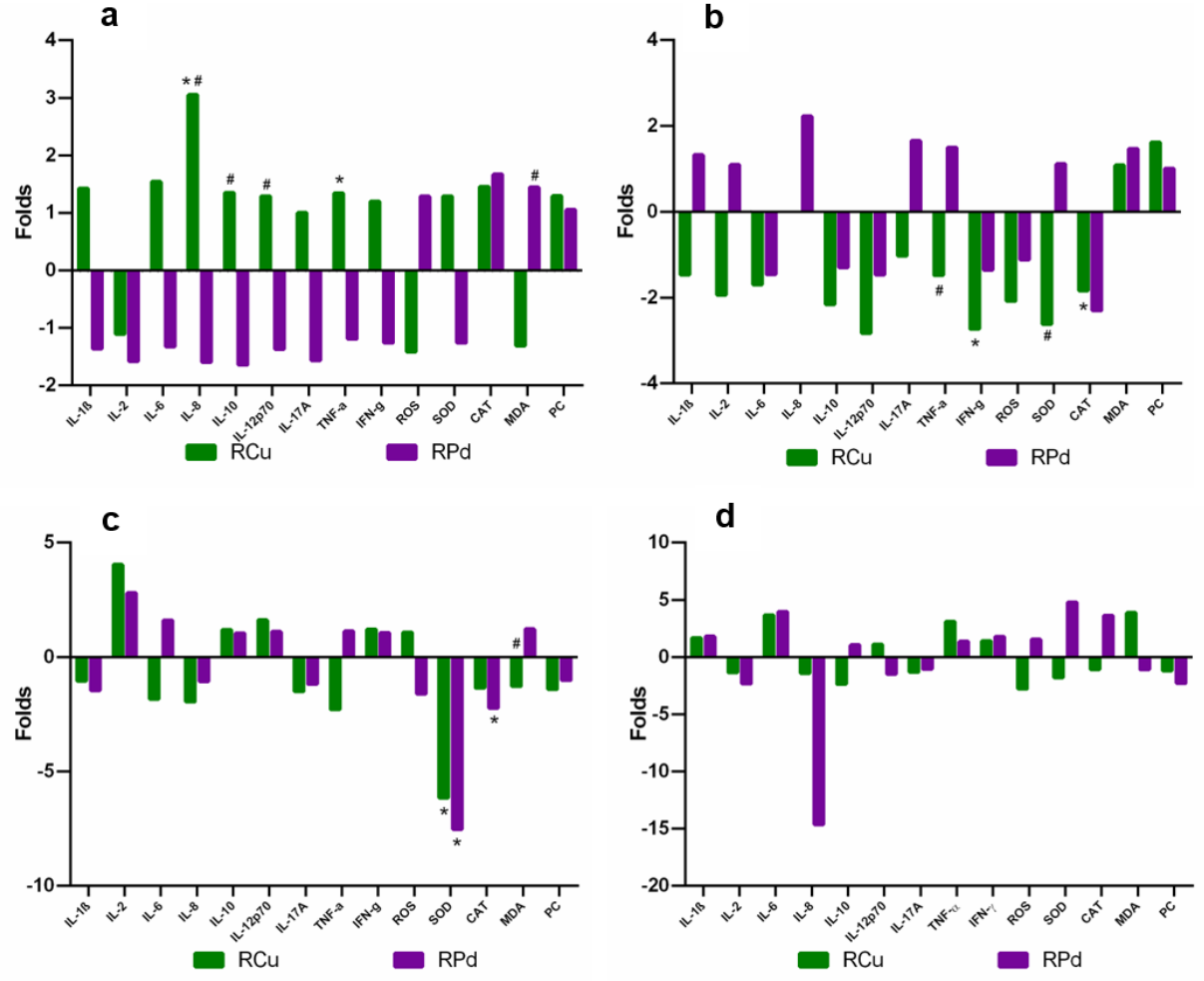

Figure 2: Comparing the cytokine and oxidative stress biomarkers of the RCu and RPd groups, respectively to the levels of the RX group in the kidneys (2a), liver (2b), serum (2c), and the tumour (2d). The comparisons are expressed as enhancement in folds. Asterisk (*) indicates statistical difference (p<0.1) to control (RX) group; hash (#) indicates statistical difference (p<0.1) between the treatment groups

As both cytokines and oxidative stress biomarkers are influenced and of relevance in cancer, it is necessary for a correlation to be found between these two. In Chapter 2, the relationship between these markers was described using different diseases, but a direct or specific correlation could not be made with cancer as yet.

After a literature search, a possible link between these cytokines and oxidative stress is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which is present in the pathways of both the markers and cascades. An analysis of NF-kB will possibly give a clearer indication of a correlation between the secondary response (oxidative stress biomarkers) and the immune response (cytokines).

The aim of this study was been achieved — from the results obtained from the study, it can be concluded that the current radiolabelled compounds do not have a significant effect on either the cytokine or oxidative stress biomarker levels of the exposed animals. However, the opposite

changes that were seen between the treatment groups need a more in-depth investigation. This study has shown that the use of non-traditional preclinical tests has a place in the development of new novel compounds.

4.2 Future recommendations

There are several recommendations for using cytokines and oxidative stress biomarkers to increase the efficiency of this study, as well as to obtain data that will be of more relevance and more representative of the effects these compounds have on humans.

- A large consideration is the manner in which the samples are collected and stored. Sufficient procedural optimisation should be done on how sampling should take place and how different markers will require different ways or methods of sampling, which is a major concern; reliable analysis cannot take place if the sampling or storing of samples was done incorrectly. For example, to determine molecular markers, snap freezing of samples with liquid nitrogen would be advisable.

- As biomarkers are time sensitive, it is important to look at other ways to evaluate these biomarkers or cytokine levels over time. For instance, each cytokine has a different response time or half-life time, which means that taking one sample after euthanasia is not sufficient to make scientific conclusions. It would rather be advisable to prepare serum samples from blood withdrawn from the animals during several different time points after exposure of the compounds, as each marker will differ at every time point. Only then can we conclude the true effect that these compounds have on individual cytokines or oxidative stress biomarkers. It will also then be possible to see how each marker responds to the compound over time and to determine the effect of these markers on each other.

- For analysis of the biochemical responses, it will be advisable to investigate a broader array of responses or markers such as the specific cytochromes involved in drug metabolism, with specific reference to the excretion or metabolism of the drug (specific organs, specific enzymes).

- As part of the above recommendation, testing of metallothionein will also contribute to a better understanding of the body's response to the metal compounds, as this is a marker in the body's reaction against metals.

- More specific toxicology studies such as acetylcholinesterase inhibition, comet assay for DNA damage, and cytochrome P450s can also be employed to see whether the bio-distribution data and specific biomarkers can be correlated. Furthermore, analyses such as the ALT: AST ratio may be useful as liver health markers.

- An essential recommendation for future studies will be to have bio-distribution data available when analysing samples other than serum or plasma, to give a better understanding of the data and levels of certain markers in specific organs, as well as to identify which organs will be the best representatives. As the spleen plays an important role during immune responses, it will be advisable also to investigate the levels of the biomarkers of the spleen. This will help to evaluate whether correlations occur between specific responses in cytokine as well as oxidative stress biomarkers in the organs of relevance, as well as the role of accumulative changes.

- Even though ARRIVE guidelines should be followed in animal studies, it would be better to use a larger number of animals (n), as variables do occur in biological samples; and, in a bigger group, outliers will have a smaller impact on the values and give a more precise representation of the actual effects on markers.

- In preclinical studies, it is also of importance to study different gender groups in their specific groups, since gender differences will be responsible for different levels of cytokine levels as well as the oxidative stress biomarkers' levels.

- It is advisable that studies containing cytokine analysis should rather be conducted in mice, as there are kits locally available for mice cytokine analysis, but not for rats at this point in time. Technical support may be needed and local support will make the process more convenient.

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Tarrant, J.M. 2010. Blood cytokines as biomarkers of in vivo toxicity in preclinical safety assessment: considerations for their use. *Toxicological sciences*, *117*(1):4 - 16.

APPENDICES

Appendix A: Ethics approval

Appendix B: Additional information on the animal study and group allocations

Appendix C: Author guidelines for the *Journal in Immunology Research*

Appendix D: Author guidelines for the *Journal of Pharmacological and Toxicological Methods*

Appendix E: Conference attendance

Appendix F: Declaration of language editor

APPENDIX A: ETHICS APPROVAL

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Health Sciences Ethics Office for Research, Training and Support

Animal Care, Health and Safety in Research Ethics Committee (AnimCare)
Tel: 018 299 2234
Email: Tiaan.Brink@nwu.ac.za

Prof AF Grobler
Pharmaceutical sciences
DST/NWU Preclinical Drug Development Platform (PCDDP)

29 November 2018

Dear Prof Grobler

APPROVAL OF YOUR APPLICATION BY THE ANIMCARE COMMITTEE OF THE FACULTY OF HEALTH SCIENCES

Ethics number: NWU-00178-18-S5

Kindly use the ethics reference number provided above in all future correspondence or documents submitted to the administrative assistant of the Animal Care, Health and Safety in Research Ethics Committee (AnimCare).

Study title: The effects of radiolabelled agents, used in the diagnosis and treatment of cancer, on inflammatory cytokines

Study leader: Prof AF Grobler

Student: H Griessel-24234052

Application type: Single study

Project Category *(impact on animal wellbeing)*	**NA**	**0**	**1**	**2**	**3**	**4**	**5**
					X		

Expiry date: 30 November 2019 ((monitoring report is due at the end of November annually until completion)

You are kindly informed that after review by the AnimCare committee, Faculty of Health Sciences, North-West University, your ethics approval application has been successful and was determined to fulfil all requirements for approval. Your study is approved for a year and may commence from 29/11/2018. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation. A monitoring report should be submitted two months prior to the reporting dates as indicated i.e. annually for Category 0-4 studies, six-monthly for category 5 studies, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimMonitoring@nwu.ac.za. Annually, a number of studies may be randomly selected for an internal audit.

The AnimCare committee, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare committee, Faculty of Health Sciences prior to implementing these changes. These requests should be submitted to Ethics-AnimCare@nwu.ac.za with a cover letter with a specific subject title indicating “Amendment request: NWU-XXXXX-XX-XX”. The letter should include the title of the approved study, the names of the researchers involved, the nature of the amendment/s being made (indicating what changes have been made as well as where they have been made), which documents have been attached and any further explanation to clarify the amendment request being submitted. The amendments made should be indicated in yellow highlight in the amended documents (or in the fillable MSWord format application forms where a yellow highlighter may not be visible, change the text

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Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form to Ethics-AnimCareIncident-SAE@nwu.ac.za. The *e-mail*, to which you attach the documents that you send, should have a specific subject line indicating that it is a notification of a serious adverse event or incident in a specific project e.g. “SAE/Incident notification: NWU-XXXXX-XX-XX”.

Please note that the AnimCare committee, Faculty of Health Sciences has the prerogative and authority to ask further questions, seek additional information, require further modification or monitor the conduct of your research. The AnimCare committee, Faculty of Health Sciences reserves the right to visit sites where approved studies will be conducted and any animal housing facility under the authority of NWU as often as it deems necessary, either announced or unannounced.

The AnimCare committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the South African National Standard (SANS) document 10386:2008 entitled, “The care and use of animals for scientific purposes”, the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely

Prof Christiaan B Brink

Chair: AnimCare

Prof Minrie Greeff

Head: Ethics Office

Current details: (13009230) G:\My Drive\9.5.1.1_Ethics\2018\NWU-00178-18-S5 \9.1.5.4.1_ Approval Letter_AnimCare.docm
29 November 2018

File reference: 9.1.5.4.1

APPENDIX B: ADDITIONAL INFORMATION ON THE ANIMAL STUDY AND GROUP ALLOCATIONS

This appendix shows and explains the study, as well as the course and planning of this study.

Animal study

The animal study consists of multiple segments. In segment 1, a baseline of the selected inflammatory cytokines and biomarkers was established in healthy RNU nude rats, which experienced the same stress as the xenografted rats. During segment 2, a reference range of the inflammatory markers and biomarkers relevant to the cancer was established, when the rats were inoculated with A549 (NSCLC) cell line, and the tumours reached a tumour volume of more than 100 mm^3. During segment 3, the compounds were administered to the rats. The rats were scanned for radiolabel distribution via microPET imaging for the major study (NWU-00251-17-A5). In segment 4, 24 hours after the compounds were administered, the rats were euthanised and dissected, followed by the radioactivity counting of the blood and tissue samples. Segment 5 included the analysis and determination of the effect that the administered compounds have on the relevant inflammatory cytokines as well as on several biomarkers.

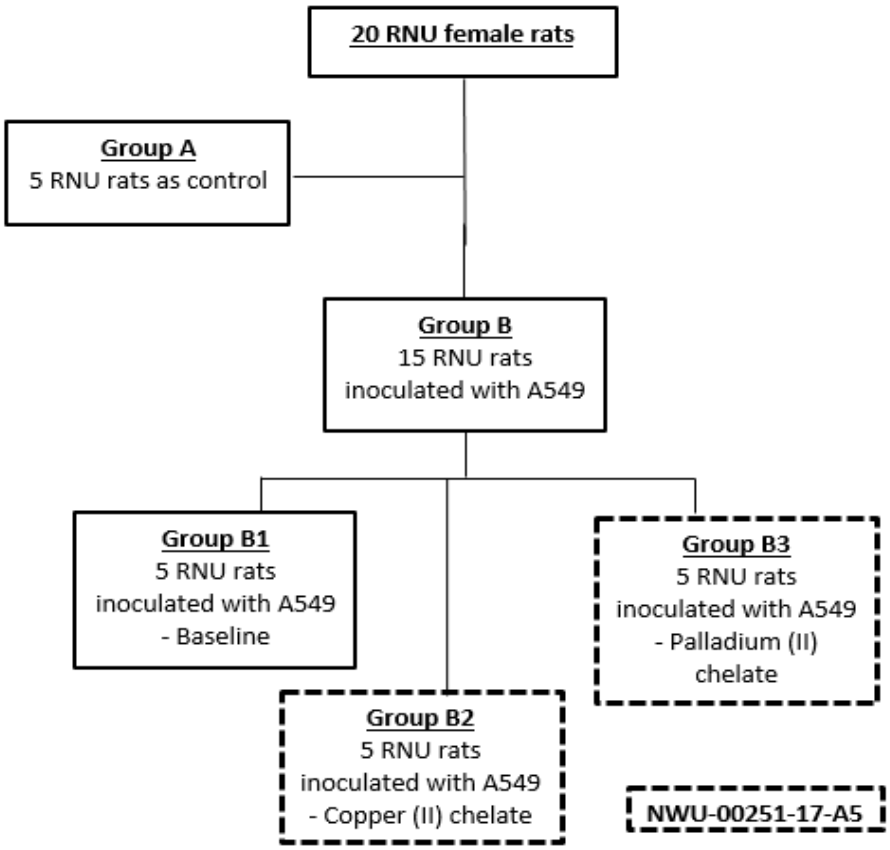

Figure 1: The sample layout explaining the experimental groups of the study

Twenty (n = 20) four-to-six-week-old female nude RNU rats were used in this study (Figure 1). Rats aged between four and six weeks are ideal for inoculation of cell lines, as rejection of the xenografts is more likely in older animals (Suckow *et al.*, 2006). Inflammatory markers differ between genders. In order to cancel out this variance, only females were used — the larger study NWU-00215-17-A5 used only female rats. The rats were randomly divided into four groups of five per group (n = 5).

Control group (Group A)

Group A contains five (n = 5) female RNU rats that were not inoculated and were not treated with the experimental compounds. This group was euthanised with isoflurane followed by decapitation and dissection.

Xenograft groups (Group B)

Three groups of five rats each were enrolled in Group B. A total of 15 rats (5 per sub-group) were inoculated with the human non-small-cell lung carcinoma cell line, A549. Group B1 was inoculated with A549, but did not receive any radiolabelled compound. They served as the baseline rats for the NSCLC (A549) inoculated rats — a cancer control. Groups B2 and B3 consisted of five rats each that were inoculated with A549 and were treated with the experiment compounds, respectively. Group B2 received the copper (II) chelate, and received the palladium (II) chelate compound.

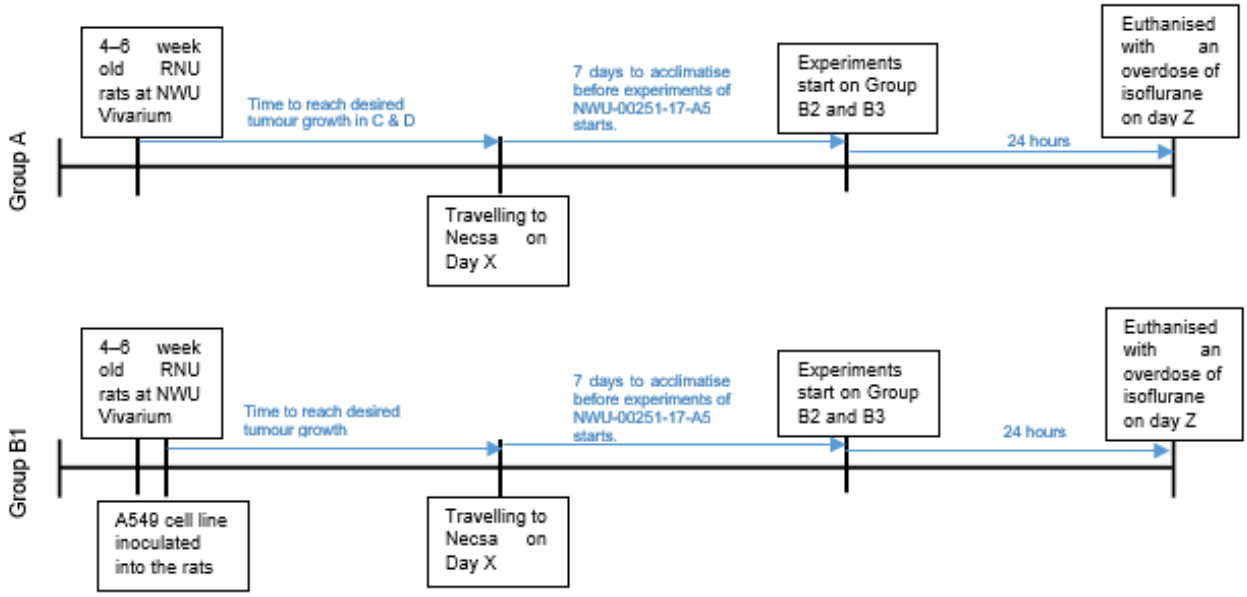

Figure 2: Timeline for study NWU-00178-18-A5 (current study)

Group B1 was inoculated with cell line A549. The tumours were given the appropriate time to reach the desired tumour size, as also mentioned for the previous figure. When the desired tumour sizes were reached, Groups A and B1 were transported to Necsa (Figure 2).

Group B1 received anaesthesia at the same time points as Group B2. Both Groups A and B1 were euthanised at the same time points as the rats of study NWU-00251-17-A5. The animals were euthanised by an overdose of isoflurane on the same day (day Z) as Groups B2 and B3 (Figure 2).

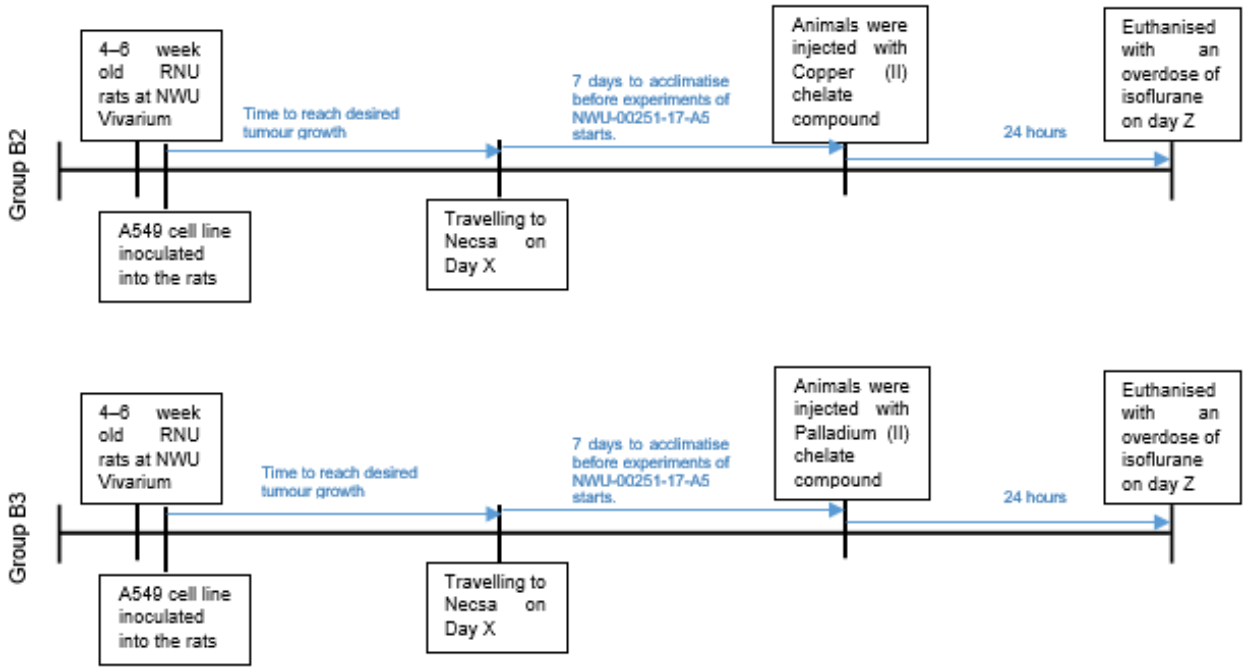

Figure 3: Timeline for study NWU-00251-17-A5

Figure 3 illustrates the timeline for study NWU-00251-17-A5. RNU rats were inoculated with the cell line — A549 — at the ages of between four and six weeks. Sufficient time passed for the tumours to grow to the desired volume of between 100 and 150mm^3. Once the tumour sizes have been reached, the animals were transported to Necsa on day X (Figure 1 & Figure 2). On arrival at Necsa, the rats were given seven days to acclimatise before any experiments started. Groups B2 and B3 were administered with copper (II) chelate compound and palladium (II) chelate, respectively, followed by imaging (NWU-00251-17-A5). Twenty-four hours after the administration of the chelated compounds, the treated animals were euthanised by an overdose of isoflurane.

APPENDIX C: AUTHOR GUIDELINES FOR THE JOURNAL IN IMMUNOLOGY RESEARCH

Guidelines for the publication in the *Journal of Immunology Research* (Impact factor 3.404)

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- STREGA for genetic association studies
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Authors may appeal if they feel that the decision to reject was based on: i) a major misunderstanding over a technical aspect of the manuscript, or ii) a failure understand the scientific advance shown by the manuscript. Appeals requesting a second opinion without sufficient justification will not be considered. To lodge an appeal, please contact the journal by email, quoting your manuscript number. Appeals will only be considered from the original submitting author.

APPENDIX D: AUTHOR GUIDELINES FOR THE JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS

Guidelines for the publication in the *Journal of Pharmacological and Toxicological Methods* (Impact factor 2.679; ISSN: 1056-8719)

JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS

AUTHOR INFORMATION PACK

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ISSN: 1056-8719

DESCRIPTION

Journal of Pharmacological and Toxicological Methods publishes original articles on current methods of investigation used in **pharmacology** and **toxicology**. Pharmacology and toxicology are defined in the broadest sense, referring to actions of drugs and chemicals on all living systems. With its international editorial board and noted contributors, *Journal of Pharmacological and Toxicological Methods* is the leading journal devoted exclusively to experimental procedures used by **pharmacologists** and **toxicologists**.

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AUDIENCE

Pharmacologists, Toxicologists, Biochemists.

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GUIDE FOR AUTHORS

INTRODUCTION

Journal of Pharmacological and Toxicological Methods publishes articles on methods used in pharmacology, safety pharmacology and toxicology. *Journal of Pharmacological and Toxicological Methods* is the leading international journal devoted exclusively to the elaboration and validation of experimental methods.

Please visit our Pharmacology Author Resources page for guidance on manuscript preparation.

Types of paper
The *Journal of Pharmacological and Toxicological Methods* publishes papers in a range of categories:

"Research paper": description and characterization of a new or modified disease model, method, technique, apparatus or approach to analysis of data. This may include interspecies comparisons that contrast drug actions from a model relevance perspective; "Short communication" detailing simple modifications of an existing model, method, technique, apparatus or approach to analysis of data; "How To" articles that provide step-by-step guidance on the execution of a specific technique; "Appraisal of state-of-the-art" or "Historical review" of particular models, methods, techniques or apparatus; "Methods in drug discovery" - perhaps the most important category. Here, by showing how a method was used to select a drug that was eventually found to be clinically effective, the author establishes validation of the method.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

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BEFORE YOU BEGIN

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Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

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Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

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Note that papers focused on the actions of drugs are beyond the scope of the journal.

Specific instructions for regular articles & short communications :

Introduction

This must outline the reason for the study and justify the approach taken.

Methods

This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Results

This section should be concise and must not contain repetition of the methods. Data in the text must not replicate data in tables or figures. SI units must be used.

Discussion

The potential value of the data to pharmacological or toxicological or safety pharmacology research methods must be clearly explained, with appropriate reference to existing methods and their limitations. This section must not contain paragraphs dealing with topics that are beyond the scope of the study. Use subheadings for clarity.

Specific instructions for "How To" articles:

" How To" articles provide step-by-step guidance on the execution of specific techniques.

Introduction

For "How To" articles, this section will be very brief, and will simply identify the therapeutic area, the goal of the method, and give mention to published alternatives descriptions if available. It is unlikely that many papers will be cited in this section.

Methods

This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Methods
This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Results

This section should be concise and must not contain repetition of the methods. Data in the text must not replicate data in tables or figures. SI units must be used.

Discussion

The potential value of the data to pharmacological or toxicological research methods must be clearly explained, with appropriate reference to existing methods and their limitations. This section must not contain paragraphs dealing with topics that are beyond the scope of the study.

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Appraisal articles are reviews about the current best models. The article should identify and describe the current best model, and discuss the evidence (or lack of) to support this. A good model should demonstrably detect drugs that work (or cause adverse effects) in humans, and demonstrably have

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Text to be divided into sections according to author choice.

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Text to be divided into the following sections

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This must not exceed 250 words and should be subdivided into four subsections headed: Introduction, Methods, Results, Discussion.

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Immediately after the abstract, provide a maximum of 10 keywords (one of which should be "methods") in alphabetical order, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, " and" , " of"). These keywords will be used for indexing purposes.

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Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163*, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.
Reference to a journal publication with an article number:
Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2018). The art of writing a scientific article. *Heliyon*, *19*, e00205. https://doi.org/10.1016/j.heliyon.2018.e00205.
Reference to a book:
Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter 4).
Reference to a chapter in an edited book:
Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S. Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing Inc.
Reference to a website:
Cancer Research UK. Cancer statistics reports for the UK. (2003). http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ Accessed 13 March 2003.
Reference to a dataset:
[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1. https://doi.org/10.17632/xwj98nb39r.1.

Reference to a conference paper or poster presentation:
Engle, E.K., Cash, T.F., & Jarry, J.L. (2009, November). The Body Image Behaviours Inventory-3: Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales. Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies, New York, NY.

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APPENDIX E: CONFERENCE ATTENDANCE

Drug Safety Conference – November 2018, Potchefstroom, South Africa

Journal of Pharmacological and Toxicological Methods 98 (2019) 106608

Contents lists available at SciVerse ScienceDirect

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Drug Safety Africa Meeting Abstracts

008

Meeting abstracts

The use of cytokines as indicators for drug safety

Heléne Griessel[a], Wihan Pheffer[a], Jesslee du Plessis[b], Jan-Rijn Zeevart[a,c], Anne Fredrica Grobler[a]
[a]DST/NWU Preclinical Drug Development Platform, South Africa
[b]Medicine Usage of South Africa, South Africa
[c]The South African Nuclear Energy Corporation, South Africa

In developing new compounds, questions about safety and efficacy are raised. Conventional preclinical studies of new compounds focus on bio-distribution, toxicology, safety pharmacology, pharmacokinetics, efficacy etc., but little information regarding the effect of these compounds on inflammatory cytokines are available. Cytokines can be used as markers to identify drug safety, focusing on cardiovascular, neurologic, and respiratory functions. Cytokines are produced by different cell groups, mainly by macrophages and helper T cells[3]. Cytokines can be divided into two main groups: anti-inflammatory cytokines and pro-inflammatory cytokines. Cytokines function as a complex network to control each other's responses and production[4]. According to literature, a dysregulation of inflammatory markers or biomarkers can be indicative of chronic inflammation [1,2]. There is no data available on the inflammatory cytokine values in healthy people and there is no formal reference of normal ranges of inflammatory markers. By establishing a baseline, it can be used in preclinical studies to determine the effects of new compounds on cytokines. The aim of this study is to determine baseline inflammatory cytokines concentration in an established *in vivo* model to eventually determine the effects of new radiolabeled compounds on the cytokines. Using an established xenograft model, a control baseline, a disease control (cancer), and drug treatment (radiopharmaceutical) cytokine levels will be determined by cytokine analyses of collected blood serum and tissues Antigenix America Inc.'s SUPER –X PLEX™ Flow Cytometry cytokine assays will be used on the BD Accuri® C6 Flow Cytometer. This multiplex assay has multiple bead populations, which are differentiated by the levels of fluorescence intensity and size. This allows for a distinct bead populations on the flow cytometer data output. It also makes it possible to measure multiple analytes with a single reaction. The following results are anticipated: Determining the individual baseline concentrations of the inflammatory cytokines. To observe a clear change in cytokine profiles between the baseline, disease control and drug treatment.

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doi:10.1016/j.vascn.2019.07.008

009

Meeting abstracts

Development of a batch process to produce ethylated fatty acid esters

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Pharmaceutical excipients account for the majority of the volume of administered drugs [1]. The problem with the manufacture of pharmaceutical excipients is that they were considered to be inert support material with no real contribution to the efficacy of drugs. Conversely, excipient formulation has proved to be a science on its own as it can significantly influence drug delivery in terms of stability, bioavailability and transfer of critical impurities and may even lead to adverse effects [1]. All manufacturers of pharmaceutical and complementary products have to be compliant with an extensive list of Good Manufacturing Practice (GMP) guidelines in order to be certified. However, the guidelines are only specified for the manufacturing process and not for each individual excipient [2]. Proper quality control has to be performed not only on the finished product, but on the raw material procurement process as well. A defined mixture of ethylated fatty acid esters used as a key component in the manufacture of the Pheroid® drug delivery system, is only available in South Africa on import. This project therefore aims to develop a sustainable batch process to produce the mixture of fatty acid ethyl esters locally, with the benefit of improving the economic feasibility of the drug delivery system. The product will be of pharmaceutical grade and the manufacturing facility will be GMP compliant. Transesterification reactions with pure ethanol and anhydrous sodium hydroxide are conducted in an attempt to optimize conversion of triglycerides to ethyl esters. Deuterated chloroform is used as solvent in NMR spectroscopy to determine the reaction conversion. The parameters to be optimized include reaction time, catalyst, concentration, reaction temperature, and molar ratio of alcohol to oil. With the optimized conditions, methods for effective separation of the product and by-products will be tested. This includes a dry method using a magnesium silicate to remove impurities and a water washing method followed by drying with anhydrous calcium chloride. In both cases, excess alcohol will be removed by distillation. The established method will be applied to larger batches in a stirred tank which will be designed as part of the study. The feedstock was characterized in terms of water and free fatty acid contents as these two parameters necessitates pre-treatment of the feedstock if it is higher than 1% and 0.5%, respectively. The water content was found to be 0.03% and the free fatty acid content 1.20%. This indicates that pre-treatment of the feedstock will be necessary.

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doi:10.1016/j.vascn.2019.07.009

The use of cytokines as indicators of drug safety

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Introduction

In developing new compounds, both safety and efficacy are critical. Conventional preclinical studies of new compounds focus on biodistribution, toxicology, safety pharmacology, pharmacokinetics, efficacy etc., with little attention paid to the effect of these compounds on cytokines. We propose that cytokines be used as markers to assess drug safety, focusing on cardiovascular, neurologic, and respiratory functions.

Cytokines are produced by different cell groups, mainly by macrophages and helper T-cells.[1] Two main groups of cytokines are found in the body: anti-inflammatory and pro-inflammatory cytokines function as a complex network to control each other's responses and production.[2] A dysregulation of inflammatory markers or biomarkers can be indicative of chronic inflammation in organs.[3,4]

Aims of the study: (i) to determine baseline inflammatory cytokines' values in an established *in vivo* model, and (ii) to determine the effects of new radiolabeled compounds on cytokines.

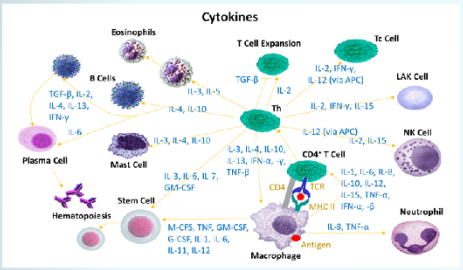
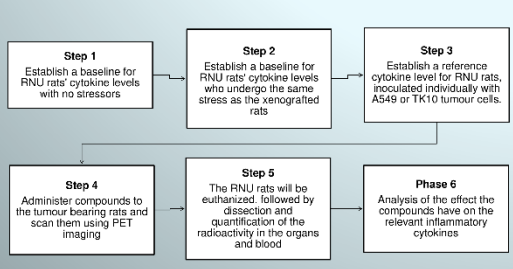


Figure 1: Expression of cytokines by various cell types.[5]

Materials and Methods

The cytokines present will be determined in blood serum and tissues of the following three groups of nude rats, using an established xenograft model:
Group 1: diseased but untreated
Group 2: diseased and treated with radiopharmaceutical and
Group 3: a healthy group of nude rats.



Materials & methods (continued)

Antigenix America Inc.'s SUPER –X PLEX™ Flow Cytometry cytokine assays will be used on the BD Accuri® C6 Flow Cytometer. This multiplex assay has multiple bead populations, which are differentiated by the levels of fluorescence intensity and size. These characteristics enables partitioning of distinct bead populations, making it possible to measure multiple analytes with a single reaction.

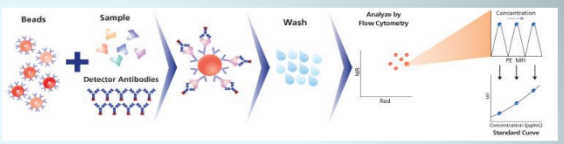


Figure 2: Process of flow cytometry analysis of cytokines[6]

Results

The following results are anticipated:
Individual baseline concentrations for the inflammatory cytokines
Clear and change in cytokine profiles after treatment

Conclusions

The development of new diagnostic and therapeutic compounds for the treatment of cancer requires an investigation of the safety and efficacy of compounds, which may also be reflected by the cytokine profile or status of an individual. The effect of new compounds on the inflammatory markers in existing animal xenograft models is being studied. An immune approach to efficacy in the treatment of cancer is especially important in immunotherapy.

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Acknowledgments

The DST/NWU Preclinical Drug Development Platform for financial assistance and Prof Faans Steyn for assisting with the statistician data

Poster presentation and Junior Investigator travel award recipient

The Use of Cytokines and Oxidative Stress Markers as Indicators for Drug Safety

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Introduction

The annual increase in cancer cases underlies the urgency of developing improved drugs and devices for the treatment and diagnosis of cancer[1,2]. Safety and efficacy are priorities when developing new drugs. Cytokines are potential markers of drug safety in physiological important systems[3]. In addition, oxidative stress markers may indicate the impact of drugs on physiological processes[4]. Cytokines or oxidative stress biomarkers can be indicative of chronic inflammation, cancer and other diseases[5-8]. A change in these markers, due to exposure to new compounds, may negatively affect the immune responses/modulation in physiological process. There is a paucity on the behaviour of inflammatory cytokines in combination with oxidative stress conditions after exposure to new cancer medication[9-13]. By establishing a baseline for preclinical safety studies *in vivo*, the effects of new compounds on oxidative stress markers and cytokines can be quantified.

The aim of this study was to determine possible changes in the cytokines and oxidative stress biomarkers in the renal and hepatic systems *in vivo*, using a xenograft rat model after treatment with novel radiolabelled compounds (confidential and under development, referred to as Cu-compound and Pd-compound).

Materials and Methods

Xenografted (A549) Rowett's nude rats were used as model. Tissue samples were collected from a control group and two radiolabelled compound treated groups of rats. Selected cytokines were analysed using a cytometric bead array (CBA) assay and flow cytometry. Fluorospectrophotometry was used to quantify the following oxidative stress biomarkers: intracellular reactive oxygen species (ROS)[14], superoxide dismutase (SOD)[15], catalase (CAT)[16,17], protein carbonyls (PC)[18], and lipid peroxidation (malondialdehyde, (MDA))[19]. Redundancy analysis (RDA) was performed, using Canoco version 5.

Results and Discussion

There were no significant changes in the intracellular ROS, MDA, or PC ($p<0.1$) in any of the samples (Fig 1A-C). In the kidney samples, the Cu-treated group had a significant increase ($p<0.1$) in IL-8 and TNF-α, and in the liver the Cu-treated had a significant decrease ($p<0.1$) in IFN-gamma (IFN-γ). In the Pd-treated group, there were no cytokines that differed significantly from the xenografted control group of rats (Fig 1A-C). However, there were significant decreases ($p<0.1$) in the oxidative stress biomarkers SOD and CAT (Fig 1B,C).

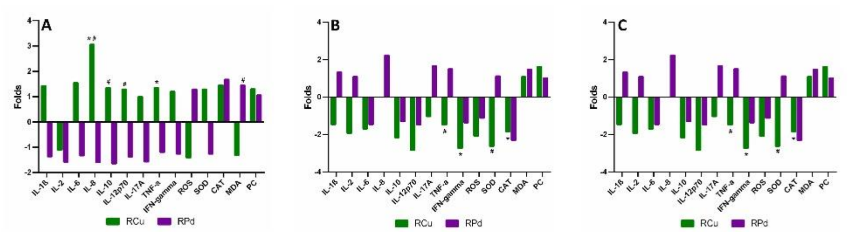

Figure 1: Fold changes in the cytokine levels and oxidative stress biomarkers of the Cu-compound (RCu) and Pd-compound (RPd) treated groups in terms of the xenograft control, in the kidney (A), liver (B), and serum (C)

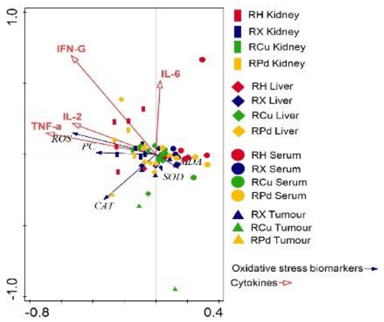

Figure 2: Redundancy analysis (RDA) of the cytokine levels and oxidative stress biomarkers in the samples from RNU xenograft rats of a control and treated groups; Cu-compound (RCu) and Pd-compound (RPd)

An RDA was performed to investigate the variance in the data based on the relationships between the cytokines and oxidative stress biomarkers (Fig 2). The explanatory variables account for 14% of the variation in the data. Factor 1 (11%) distinguishes between the pro-inflammatory (left ordination) and anti-inflammatory markers (right ordination). The variation in data is statistically ($p<0.05$) explained by the markers represented in the graph. A positive correlation was found between TNF-α, IFN-γ, IL-2 and ROS. The RDA reveals that the highest response to drug exposure were in the kidneys, whereas the least response was in the serum. The ordination of SOD correlates with the statistical changes seen in the serum (Fig 1C).

These results show that exposure to or treated with a specific radiolabelled compound did not induce oxidative stress. However, between the exposure groups, there was an opposite reaction in the kidneys' cytokine responses — an increase for Cu and decrease for Pd — of which IL-8, IL-10 and IL12p70 were significantly different ($p<0.1$). This indicates an overall pro-inflammatory response in the kidneys exposed to the Cu-compound. The phenomenon may be due to the biotransformation of the compound in view of the fact that the kidney is the main route of excretion. Future biodistrubution data will enable us to confirm this.

Conclusions

The use of cytokines is potentially a valuable preclinical drug development screening tool. As shown in the above study, a change in the inflammatory markers of physiologically important systems, such as the kidneys, is detectable after treatment with or exposure to compounds. The knowledge that there is a potential effect on the immune responses, the safety of the drug can be further scrutinised, by combining these screening tools with other specialised drug safety testing assays.

Acknowledgments

The DST/NWU PCDDP for financial contribution, Prof Faans Steyn for statistical assistance. Prof Matthew Akerman and the Necsa staff for the assistance

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Oral presentation

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Thursday, 10 October 2019		
08:15 – 09:45	**Shirra Moch**	**Workshop 2:** Selected responses in written assessment – demystifying MCQs
09:45 – 10:15	Tea break & Poster session	
10:15 – 13:00	**Young scientists**	**Session chair:** Gill Enslin
10:15 – 10:30	Yuri-Andropov Jowah	A comparative qualitative study of the medicine registration policies and processes of some National Medicines Regulatory Authorities in the Southern African Development Community
10:30 – 10:45	Yasmine Khan	Proposed antimicrobial stewardship curriculum for the South African Bachelor of Pharmacy programme
10:45 – 11:00	Kate da Silva	3D Printed, artificial extracellular matrix for potential neuroregeneration
11:00 – 11:15	Kara de la Harpe	An advanced 3D monofilament biosuture
11:15 – 11:30	Rostand Fankam Pokomi	Selection, synthesis and evaluation of novel drug-like compounds from a library of virtual compounds designed from natural products with antiplasmodial activities
11:30 – 11:45	Lara Freidus	Novel pH-triggered, fluorescent, mesoporous silica nanoparticles for potential theranostic applications in cancer intervention
11:45 – 12:00	Heléne Griessel	Immune response in xenograft rat model after the administration of novel diagnostic and treatment compounds
12:00 – 12:15	Priveledge Mazonde	Preparation, characterisation and *in vitro* assessment of efavirenz loaded flaxseed oil nano-emulsions
12:15 – 12:30	Veronica Maphanga	Screening selected medicinal plants for potential anxiolytic activity using an *in vivo* zebrafish model
12:30 – 12:45	Bjorn Martin	Formulation and characterisation of corticosteroid loaded ethosomes for topical delivery
12:45 – 13:00	Kevine Kanama	An *in vitro* multi-parameter hepatotoxic assay of selected South African medicinal plants
13:00 – 14:00	Lunch	
14:00 – 15:15	**Young scientists**	**Session chair:** Sandile Khamanga
14:00 – 14:15	Joseph Mutenga	Synthesis and characterisation of isoniazid loaded mesoporous calcium carbonate microparticles
14:15 – 14:30	Zenande Ngcauzele	Isolation and identification of ciprofloxacin resistant bacteria in Buffalo River at King Williams Town, South Africa
14:30 – 14:45	Ramoagi Segone	Rapid differentiation of *Piper methysticum* (kava) plant parts using single point and imaging vibrational spectroscopy
14:45 – 15:00	Lana Strydom	Statistical optimisation of a terbinafine cream using experimental design
15:00 – 15:15	Luke Zondagh	Design, synthesis and biological evaluation of edaravone derivatives bearing the *N*-benzyl pyridinium moiety as multifunctional anti-Alzheimer's agents

APPENDIX F: DECLARATION OF LANGUAGE EDITOR

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Language editing and translation
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24 October 2019

To whom it may concern

Dear Mr / Ms

Re: Language editing of dissertation:

The effects of radiolabelled agents used in the diagnosis and treatment of cancer, on inflammatory cytokines

I hereby declare that I language edited the above-mentioned dissertation by Ms Heléne Griessel (student number: 24234052).

Please feel free to contact me should you have any enquiries.

Kind regards

Cecile van Zyl

Language practitioner

BA (PU for CHE); BA honours (NWU); MA (NWU)
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